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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12Q 1/68, A61K 48/00, C07H 21/04	A1	(11) International Publication Number: **WO 00/53808** (43) International Publication Date: 14 September 2000 (14.09.00)

(21) International Application Number: PCT/US00/06096

(22) International Filing Date: 9 March 2000 (09.03.00)

(30) Priority Data:
09/265,630 9 March 1999 (09.03.99) US

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: BECLIN AND USES THEREOF

(57) Abstract

This invention provides a method of diagnosing a predisposition to carcinoma by determining the beclin allele status of a patient. This invention also provides uses of beclin for treating viral disease, inhibiting viral replication by administering an effective amount of Beclin with the virus infected cell, inhibiting viral replication, treating cancer by inducing increased expression of Beclin.

BECLIN AND USES THEREOF

5   This application claims priority of U.S. Serial No.
    09/265,630, filed March 9, 1999, which is a continuation-in-
    part of U.S. Serial No. 09/250,045, filed February 12, 1999,
    which is a continuation-in-part of U.S. Serial No.
    09/040,808, filed March 18, 1998, which is a continuation-in-
10 part of International Application No. PCT/US97/16358, filed
    September 12, 1997, which is a continuation-in-part of U.S.
    Serial No. 08/712,939 filed September 13, 1996, now U.S.
    Patent No. 5,858,669, issued on January 12, 1999, the
    contents of which are hereby incorporated into this
15 application by reference.

    The invention disclosed herein was made with Government
    support under Grant Nos. K08AI01217-01 and R29AI40246 from
    the National Institutes Of Health of the United States
20 Department of Health and Human Services. Accordingly, the
    U.S. Government has certain rights in this invention.

    Throughout this application, various publications are
    referenced by author and date. Full citations for these
25 publications may be found listed alphabetically near the end
    of the specification. The disclosures of these publications
    in their entireties are hereby incorporated by reference into
    this application in order to more fully describe the state
    of the art as known to those skilled therein as of the date
30 of the invention described and claimed herein.

    BACKGROUND

    Beclin is a novel, 61 kd, Bcl-2-interacting, coiled coiled
    protein encoded by a gene located on a tumor susceptibility
35 locus on chromosome 17q21. This application shows that
    Beclin has tumor suppressor and anti-apoptotic functions.
    The region of chromosome 17q21 encoding Beclin is deleted in
    50-75% of cases of prostate cancer, ovarian cancer, and
    breast cancer. This application shows that *beclin* is a tumor
40 suppressor gene important for breast, ovarian, and prostate

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cancer. The experiments herein show the presence of aberrant splice products in sporadic epithelial ovarian cancers that contain monoallelic deletions of *beclin*, and the loss of Beclin protein expression in sporadic invasive breast
5   carcinomas. These findings suggest that mutations in *beclin* splice site junctions, mutations in regulatory regions of the *beclin* gene (e.g. promoters, enhancers, 5' and 3' nontranslated regions), mutations in the coding region which may lead to decreased protein stability, and/or
10 hypermethylation of the promoter region, are important mechanisms for functional inactivation of *beclin* and subsequent tumorigenesis in breast, ovarian, and/or prostate cancer.

15 Monoallelic deletions of chromosome 17q21, detected by loss-of heterozygosity (LOH), are found in 50% of breast carcinomas, 50-75% of ovarian carcinomas, and 40% of prostate carcinomas (Cropp et al. 1993; Futreal et al. 1992; Saito et al. 1993; Eccles et al 1992; Russell et al. 1990; Sato et
20 al. 1991; Yang-Feng et al. 1993; Gao et al. 1995; Gao et al. 1995). This frequent LOH in chromosome 17q21 suggests that inactivation of one or more tumor suppressor genes located on this chromosomal arm are important for breast ovarian, and prostate carcinogenesis.   In 1994, the existence of one such
25 gene, BRCA1, was identified by positional cloning, and mutations in BRCA1 are now accepted to be an important cause of hereditary breast and ovarian cancer (Futreal et al. 1994; Miki et al. 1994).   However, it remains unclear whether functional inactivation of BRCA1 represents an important
30 molecular event in the development of sporadic breast and ovarian carcinomas. (Vogelstein and Kinzler 1994; Tangir et al. 1994).   To fulfill the Knudson two-hit hypothesis (Knudson 1985) for inactivation of a tumor suppressor gene as a mechanism for oncogenesis, a mutation has to be found
35 in the remaining allele of tumors showing *BRCA1* locus deletion.   To date, somatic mutations in *BRCA1* have been found in very few cases of sporadic cancers (Futreal et al.

1994; Hosking et al. 1995; Merajver et al. 1995; Takahashi et al. 1995).

5   In further support of this hypothesis, more finely detailed deletion mapping has identified a 400 kb deletion unit approximately 60 kb centromeric to the BRCA gene in sporadic epithelial ovarian cancer (Tangir et al. 1996). This deletion unit may contain a putative tumor suppressor important for sporadic ovarian cancer and contains
10 approximately 12 genes which were identified during the search for BRCA1. Six of them are known genes or human homologs of other species; gamma tubulin, homolog of D. melanogaster enhancer of zeste, pseudogene of HMG17, homology of Pacific electric ray VAT1, glucose-6-phosphatase and Ki
15 antigen and six of them are novel genes.

This application describes the identification of the full-length open reading frame and functional characterization of one of these six novel genes, which is
20 herein referred to as "beclin". This application shows that Beclin overexpression inhibits cellular proliferation in vitro and suppresses the ability of human breast carcinoma cells to form colonies in soft agar and tumors in nude mice. Thus, these studies demonstrate that beclin has tumor
25 suppressor function. This application describes the sequences of the 5' and 3' untranslated regions of beclin, and provides evidence that beclin may be functionally inactivated in human cancers by mutations in the gene which lead to loss of Beclin protein expression or by splice site
30 mutations which lead to aberrant beclin splicing.

The cellular antiapoptotic gene bcl-2 represents a novel class of antiviral host defense molecules which function both by restricting viral replication and by preventing virus-
35 induced cell death. Bcl-2 blocks apoptosis in vitro induced by several different RNA viruses, including Sindbis virus,

influenza virus, reovirus, Semliki Forest virus, LaCrosse
virus, and Japanese B encephalitis virus. Previously, we
have shown that Bcl-2 overexpression in virally infected
neurons in vivo also protects mice against fatal encephalitis
5   caused by the prototypic alphavirus, Sindbis virus. The
protective effects of Bcl-2 against fatal Sindbis virus
encephalitis were associated with a reduction both in
neuronal apoptotic death and in central nervous system (CNS)
viral replication. A similar antiviral effect of Bcl-2
10   overexpression has been observed during Sindbis virus
infection in cultured AT3 cells as well as during influenza
virus infection of MDCK cells, Japanese B encephalitis virus
infection of N18 cells, and Semliki Forest virus infection
of AT3 cells. Although the role of endogenous Bcl-2 in
15   antiviral defense has yet to be evaluated, these studies
support the hypothesis that Bcl-2 may be important in
protecting cells against viral infections.

The process of autophagy, or bulk degradation of cellular
20   proteins through an autophagosomic-lysosomal pathway, is
important in normal growth control and may be defective in
tumor cells. However, little is known about the genetic
mediators of autophagy in mammalian cells and whether genetic
mediators of autophagy influence tumor development.
25   Recently, 14 genes, the *apg* genes, have been identified in
S. cerevisiae that are required for yeast autophagy[1]. The
mammalian gene encoding Beclin 1[2], a novel Bcl-2-interacting,
coiled-coil protein, shares structural similarity with the
yeast autophagy gene product, Apg6/Vps30p[3,4], and is
30   monoallelically deleted in 40-75% of sporadic human breast
cancers and ovarian cancers [5-11]. Here we show, using gene
transfer techniques, that *beclin 1* promotes autophagy both
in autophagy-defective yeast with a targeted disruption of
apg6/vps30 and in autophagy-defective human MCF7 breast
35   carcinoma cells. The autophagy-promoting activity of *beclin
1* in MCF7 cells is associated with an ability to inhibit MCF7

cellular   proliferation,   *in   vitro*   clonigenicity,   and
tumorigenesis in nude mice. Furthermore, endogenous Beclin
1 protein expression is frequently undetectable or low in
malignant human breast epithelial cell lines and tissue, but
5   is expressed ubiquitously at high levels in normal breast
epithelia. Thus, *beclin 1* is a mammalian autophagy gene with
tumor suppressor function that is expressed at decreased
levels in human breast cancer. These findings indicate that
there is an evolutionarily conserved genetic link between
10 autophagy and tumor suppressor pathways, and raise the
possibility that decreased expression of autophagy proteins
may contribute to the development or progression of breast
and other human malignancies.

15 Autophagy is an evolutionarily conserved process that occurs
in both yeast and mammalian cells in which there is bulk
degradation   of   cellular   contents   via   an
autophagosomal-lysosomal pathway. The process of autophagy
liberates free amino acids and nucleotides and enable cells
20 to survive under nutrient deprivation conditions as well as
to undergo structural remodeling during differentiation.
Protein degradation through an autophagy pathway has also
been postulated to serve as a mechanism for negative
regulation of cell growth. In support of this theory,
25 cancerous transformation *in vitro* is associated with
decreased rates of autophagic degradation. Malignant cell
lines display less autophagic activity than their normal
counterparts and are less prone to increase autophagic
activity in response to serum deprivation or high cell
30 density. Furthermore, in rat liver carcinogenesis models,
there is an inverse correlation between stage of malignancy
and autophagic activity. However, it is not known whether
reduced autophagy in tumor cells directly contributes to
deregulated cell growth, or alternatively, is the result of
35 malignant transformation.

SUMMARY OF THE INVENTION

This invention provides a method of diagnosing a predisposition to carcinoma in a subject comprising: a) obtaining a nucleic acid sample from the subject; and b)

5   determining whether one or more of the subject's beclin alleles or regulatory regions to those alleles are deleted or different from the wild type so as to reduce the subject's expression of polypeptide having tumor suppressor activity.

10   This invention also provides the above-described method, wherein one or more beclin alleles contain splice site junction mutations.

This invention provides also the above-described methods,
15   wherein the determining of step (b) comprises determining whether one or more beclin promoter regions are hypermethylated.

Further, this invention provides the above-described methods,
20   wherein the determining of step (b) comprises determining whether one or more beclin enhancer regions differ from the wild type.

This invention further provides the above-described methods,
25   wherein the determining of step (b) comprises determining whether one or more beclin 5' or 3' nontranslated regions differ from the wild type.

This invention also provides the above-described methods,
30   wherein the determining of step (b) comprises determining whether one or more beclin alleles have a premature termination codon.

This invention also provides the above-described methods,
35   wherein a majority of the nucleotides corresponding to positions 1042-1185 of figure 1B have been deleted.

Further, this invention provides the above-described methods, wherein the subject was preselected based upon an abnormality at chromosome position 17q21.

5   This invention also provides the above-described methods, wherein the nucleic acid sample is obtained from the subject's blood.

This invention further provides a method of diagnosing a
10 predisposition to carcinoma in a subject, comprising determining whether the subject has an abnormal subcellular distribution of Beclin.

In addition, this invention provides a method of diagnosing
15 a predisposition to carcinoma in a subject, comprising contacting the contents of one or more of the subject's cells with one or more antibodies or fragments thereof specific to Beclin.

20 This invention provides a method for inhibiting viral replication comprising contacting an effective amount of Beclin with the virus infected cell, thereby inhibiting the viral replication where the method for inhibiting viral replication comprising contacting induction of the expression
25 of Beclin with the virus infected cell, thereby inhibiting the viral replication.·

A method of treating a viral infection in a subject, wherein the viral infection is alleviated by the expression of Beclin
30 which comprises administering to the subject an effective amount of the pharmaceutical composition effective to express Beclin, thereby treating the viral infection in the subject.

35 In addition, this invention provides a method for treating cancer comprising inducing increased expression of Beclin, where the method for treating cancer comprises administering

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to the subject a therapeutically effective amount of beclin
so as to restore cell growth control.

This invention provides a pharmaceutical composition which
5 comprises Beclin and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A and 1B
Deduced amino acid sequence and nucleotide sequence of human
5   beclin.

Figure 1A
The deduced amino acid sequence was used to scan various data
banks. The boxed area represents the Bcl-2 binding domain
10   of human Beclin (see Table 1) and the underlined area
corresponds to the region that is predicted to have a
coiled-coil conformation.

Figure 1B
15   The nucleotide sequence of human Beclin.   The partial
nucleotide sequence of mouse Beclin obtained from sequencing
clone F1 was aligned with an overlapping clone GT197 isolated
from human breast (Rommens, 1995).   Primers immediately
upstream and downstream of the predicted open reading frame
20   were used to amlify the coding sequence of human beclin from
a normalized human infant brain cDNA library (Soares, M.B.,
et al., 1994).

Figures 2A, 2B, 2C and 2D
25   Beclin mRNA and protein expression. For Figures 2C and 2D,
cells were co-transfected with 4 µg of pSG5/human bcl-2 and
4 µg of pSG5/beclin using lipofectin and fixed after 48 hours
with 100% ETOH.   Beclin expression was detected with a
monoclonal anti-flag M2 Ab (1:20) and FITC-conjugated horse
30   anti-mouse IgG Ab and Bcl-2 expression was detected with a
polyclonal rabbit anti-Bcl-2 Ab and rhodamine-conjugated goat
anti-rabbit Ab.   Individual co-transfected cells were
analyzed with confocal laser microscopy.   In Figures 2B,
equal amounts of loading (2 µg of poly A) was confirmed by
35   hybridization to a B-actin probe.

Figure 2A

Western blot analysis of cell lysates prepared from BHK cells
infected with a recombinant Sindbis virus chimera containing
5   a flag epitope tagged human beclin insert (lane 2) or a
control recombinant Sindbis virus chimera (lane 1) and probed
with an anti-flag antibody M2 antibody (IBI) using well-known
methods .

10   Figure 2B

Northern blot analysis of beclin mRNA expression in human and
mouse tissues.   Human and mouse multiple tissue blots
(Clontech) were hybridized respectively, according to
manufacturer's instructions (Clontech) with a ^{32}P-labeled 485
15   base pair probe corresponding to nucleotides 1-485 of human
or mouse beclin.   Beclin-specific probes hybridized to 2.3
kb transcripts in all examined tissues.

Figure 2C

20   Immunofluorescence staining of human *bcl-2* in a BHK cell.

Figure 2D

Immunofluorescence staining of human *beclin* in the same BHK
cell shown in Figure 2C.

25   Figures 3A, 3B, 3C, 3D, 3E, 3F, 3G, 3H, and 3I

Interactions of Bcl-2 and Beclin *in vivo* demonstrated using
FRET microscopy.   For the experimental cells, there were
three types of labeling: acceptor and donor (n=7), donor
alone (n=10), and acceptor alone (n=10).   Each labeling type
30   was imaged using the three filter sets: the donor filter set,
the acceptor filter set, and the combination set (i.e. FRET
set) which best detects the FRET signal.   Several cells of
each type were imaged so that there were many possible
triplets of cells consisting of one each of acceptor plus
35   donor, donor alone and acceptor alone.   All possible triplets

were analyzed; triplets which generated divide by zero erros
or negative energy transfers were excluded. The same method
was applied to the control cells where there were 13 acceptor
plus donor cells, 10 donor cells alone and 10 acceptor cells
5   alone.

Figures 3A, 3D, and 3G
Donor (FITC) filter set was used.

10 Figures 3B, 3E, and 3H
FRET filer set was used.

Figures 3C, 3F, and 3I
Acceptor (Rhodamine) filter set was used.

15

Figures 3A, 3B, 3C, 3D, 3E, and 3F
Cos cells co-transfected with flag epitope-tagged Beclin and
Bcl-2, labeled with anti-flag (donor, FITC) and anti-Bcl-2
(acceptor, Rhodamine) specific antibodies, respectively.

20

Figures 3G, 3H, and 3I
Cos cells transfected with Bcl-2 labeled with antibodies
against SERCA (Research Design, Inc.)(donor, FITC) and Bcl-2
(acceptor, Rhodamine). Cos cells were co-transfected with
25 pSG5/flag-Beclin and pSG5/BCL-2, labled as in Figure 2C and
2D, and FRET microscopy was performed as described herein.

Figures 4A, 4B, and 4C

Effect of *beclin* and antisense *beclin* on Sindbis
virus-induced apoptosis. Recombinant chimeric viruses
SIN/antisense bcl-2, SIN/antisense *beclin* were constructed
using methods described for the construction of
SIN/flag-*beclin* and infected at a multiplicity of infection
(MOI) of one plaque-forming unit per cell. Cell viability
was determined by trypan blue exclusion at serial time points
after infection in Figure 4A and Figure 4B, and at 24 hours
after infection in Figure 4C. The results of triplicate
wells are shown (mean = S.E.). Similar results were obtained
in more than 5 independent experiments for Figure 4A and
Figure 4B and in three independent experiments in Figure 4C.

Figure 4A

Cell viability of BHK cell death after infection with
recombinant Sindbis viruses containing bcl-2 in either the
sense (SIN/bcl-2) or antisense orientation (SIN/antisense
bcl-2) or *beclin* in either the sense (SIN/*beclin*) or
antisense orientation (SIN/antisense *beclin*).

Figure 4B

Cell viability of AT3/Bcl-2 cells after infection with
SIN/CAT, SIN/*beclin*, SIN/antisense *beclin*, or SIN/antisense
bcl-2.

Figure 4C

Cell viability of NIH 3T3 antisense beclin and NIH 3T3
control cells after infection with Sin/bcl-2 (shaded bars)
or SIN/bcl-2 stop (hatched bars) in the presence or absence
of tetracycline. NIH 3T3 clones were cultured for three days
in the presence or absence of 1 µg/ml tetracycline prior to
infection.

Figures 5A, 5B, and 5C

Effect of beclin on AT3 and mouse fibroblast NIH 3T3 cellular proliferation. For Figures 5A and 5B, 5 x 10^5 cells were seeded in 35 mm wells, and 24 hours later, infected at a MOI of 1. Results are presented as means ± s.e. For Figure 5C, cells were cultured for three days ± tetracycline prior to seeding 5×10^5 cells/well in 96 well plates. At several time points in culture, cells proliferation was measured by performing MTT assays (MTT proliferation kit, Boehringer Mannheim) according to the manufacturer's instructions. Results are presented as mean O.D. ± s.e for triplicate wells. Similar results were obtained in three independent experiments.

Figure 5A

Determination of total cell number of AT3/bcl-2 cell number.

Figure 5B

Determination of percentage of cell viability of AT3/blc-2 cell viability after infection with SIN/CAT (open circles), SIN/beclin (solid circles) or mock infection (solid squares).

Figure 5C

NIH beclin clone 10 (circles), NIH antisense beclin clone 15 (squares) and NIH control clone 4 (triangles) proliferation in the presence (open symbols) or absence (cloned symbols) of tetracycline.

Figures 6A, 6B, and 6C

Effect of beclin transfection on the morphology of human MCF7 breast carcinoma cells.

Figure 6A

Western blot analysis of flag-Beclin expression in MVF7 clones transfected with BC252SV40/beclin. BHK cell lysates infected with a recombinant Sindbis virus expressing flag-

tagged Beclin is used as a positive control (lane 1). MCF7
Beclin clones 1, 4 and 17 (lanes 2, 3 and 4 respectively) and
a control MCF7 clone (lane 5). Clone 17, lane 4 demonstrates
Beclin immunoreactivity.

5

Figure 6B
Phase contrast micrographs of MCF7 control clone 5 cells.

Figure 6C
10 MCF7 Beclin clone 17 cells at 375 x magnification.

Figure 7
Diagram of plasmid pSG5/beclin (ATCC Accession No. 97664).
The 1.4 kb insert encoding Beclin was inserted into Eco RI
15 sites in the plasmid, pSG5 (Stratagene).

Figures 8A and 8B
Nucleotide sequence of 5' untranslated region of human beclin
(A) and 3' untranslated region of human beclin (B).

20

Figure 8A
The sequence of the insert in the plasmid TA/clone 5

Figure 8B
25 The sequence of the insert in the plasmid TA/clone 13.

Figure 9A

Schematic diagram of wild-type *beclin* aberrant *beclin* transcripts found in sporadic epithelial ovarian cancer. The clone number listed on the left of each of the three ovarian cancer splice variants refer to cloned transcripts that represent each type of aberrant splice pattern, and corresponds to the insert sequences provided in Figs. 9C-9E.

Figure 9B

Nucleotide sequence and its predicted translation of wild-type human beclin. In another polymorphic form, the nucleotide at position 308, shown as a (lower case) "c" may instead be a T, thus resulting in a valine.

Figure 9C

Nucleotide sequence and its predicted translation of mutant beclin clone 1472. In another polymorphic form, the nucleotide at position 308, shown as a (lower case) "c" may instead be a T, thus resulting in a valine.

Figure 9D

Nucleotide sequence and its predicted translation of mutant beclin clone 1259. In another polymorphic form, the nucleotide at position 308, shown as a (lower case) "c" may instead be a T, thus resulting in a valine.

Figure 9E

Nucleotide sequence and its predicted translation of mutant beclin clone 1384. In another polymorphic form, the nucleotide at position 308, shown as a (lower case) "c" may instead be a T, thus resulting in a valine.

Figure 10A and 10B

MCF7 cells were transfected with pSG5 expression vectors
containing flag epitope-tagged Beclin or flag epitope-tagged
mutant BeclinΔ1042-1185, fixed after 24 hours with 100% ETOH,
5   and subjected to immunofluorescence staining to detect
flag-Beclin expression with an anti-flag antibody M2 (VWR
Scientific).

Figure 10A

10 Subcellular localization of wild-type Beclin.

Figure 10B

Subcellular localization of mutant beclin deleted of
nucleotides 1042-1185 (BeclinΔ1042-1185).

15

Figure 11

Western blot analysis of Beclin protein expression. Western
blot analysis of human Beclin protein expression in matched
normal and tumor breast tissue from patients with sporadic
20 invasive breast carcinoma.   Twenty-five microliters of
homogenized breast tissue (diluted 1:1 in SDS sample buffer)
was subjected to SDS-Page, transferred to nitrocellulose,
probed with a polyclonal anti-human Beclin peptide (amino
acid 1-15) antibody 843(custom production by Eurogenetics,
25 Belgium), and Beclin protein was detected with ECL (Amersham)
according to manufacturer's instructions.   The positive
control represents baby hamster kidney cell lysates prepared
24 hours after infection with a Sindbis virus vector which
overexpresses human Beclin.

30

Figures 12A and 12B

Immunocytochemical analysis of Beclin protein expression in
normal breast tissue (Figure 12A) and malignant breast tissue
(Figure 12B) from patient 516 (see Figure 11 Western blot)
35 using the anti-human Beclin antibody 843 and the ABC
immunoperoxidase method.   Normal epithelial cells (Figure

12A) display marked Beclin immunoreactivity whereas malignant epithelial cells (Figure 12B) display no Beclin immunoreactivity.

5   Figure 13
Deduced amino acid sequence of human Beclin. The boxed area represents the Bcl-2-binding domain of human Beclin (Table 1), and the underlined area corresponds to the region that is predicted to have a coiled-coil conformation.

10

Figure 14
Beclin mRNA and protein expression. (A) Northern blot analysis of *beclin* mRNA expression in human and mouse tissues. Sm., small; m., muscle. (B) Immunoperoxidase
15 staining of an adult human hippocampus section with a polyclonal antibody against a human Beclin peptide. The arrow marks a Beclin-positive neuron. Magnification, X450.

Figure 15
20 Expression of flag-Beclin protein constructs by the virus vectors SIN/*beclin*, SIN/*beclin*stop, and SIN/*beclin*ΔBcl-2BD, (A and B) Western blot analyses of virus-infected BHK cell lysates with either anti-flag epitope antibody M2 (A) or polyclonal rabbit anti-Beclin antiserum (B). Lane 1,
25 SIN/*beclin*; lane 2 SIN/*beclin*stop; lane 3, SIN/*beclin*ΔBcl-2BD1; lane 4, empty Sindbis virus vector. (C to E) Immunoperoxidase stainingusing anti-flag epitope antibody M2 of mouse brains 2 days after infection with SIN/*beclin* (C), SIN/*beclin*stop (D), or SIN/*beclin*ΔBcl-2BD (E).
30 Magnification X111.

Figure 16
Confocal laser scanning microscopy of COS7 cells expressing Bcl-2 and Beclin. (A and B) Cell cotransfected with pSG5/bcl-
35 2 and pSG5/flag-beclin and stained with anti-flag epitope (A)

and anti-human Bcl-2 (B) antibodies. (D and E) Cell
cotransfected with pSG5/bcl-2 and pSG5/flag-beclinDBcl-2BD
and stained with anti-flag epitope (D) and anti-human Bcl-2
(E) antibodies. (C and F) Computerized overlays of panels
5 A and B(C) and panels D and E(F); yellow color corresponds
to overlap of FITC and rhodamine staining. Confocal slides
were 1 μm thick.

Figure 17
10 Effects of Beclin and Bcl-2 and Bcl-2 on the survival of mice
infected with Sindbis virus strain TE12. (A) Survival curve
of mice infected with SIN/*beclin*, SIN/beclinstop, and
SIN/bcl-2stop. Data represent combined survival probabilities
for three independent litters.
15

Figure 18
Viral growth of SIN/*beclin*, SIN/*beclin*stop, and
SIN/*beclin*ΔBcl-2BD in mouse brain. Each data point represents
geometric mean viral titer ± SEM of three to six mouse
20 brains.

Figure 19
Viral RNA-positive cells in mouse brains infected with
SIN/*beclin*, SIN/*beclin*stop, and SIN/*beclin*ΔBcl-2BD. Each data
25 point represents the mean ± SEM number of Sindbis virus
message-sense RNA-positive cells per square millimeter of
brain for three to six mouse brains.

Figure 20
30 Apoptotic nuclei in mouse brains infected with SIN/*beclin*,
SIN/*beclin*stop, and SIN/*beclin*ΔBcl-2BD. Each data point
represents the mean ± SEM number of ISEL-positive nuclei per
square millimeter of brain for three to six mouse brains.

35 Figure 21

Representative photomicrographs of fields used for
computerized quantitative image analysis of viral RNA (A to
C)- and ISEL (D to F)- positive cells in mouse brains
infected with SIN/*beclin* (A and D), SIN/*beclin*stop (B and E),
5 and SIN/*beclin*ΔBcl-2BD (C and F) in Fig. 19 and 20,
respectively. All photomicrographs are from the colliculus
region of brain sections 4 days after infection and
correspond to the images observed at a magnification of X10.
Arrowheads denote representative cells that were scored as
10 positive by the Image-ProPlus software program.

Figure 22
Shows the growth curve of the virus, a gammaherpesvirus 68,
in human MCF7 breast carcinoma cells that are transfected
15 with an empty vector (MCF7/control cells) or with a vector
expressing human *beclin* (MCF/*beclin* cells).

Figure 23
Complementation of *apg6/vps30* by *beclin 1* in yeast
20 autophagy. a, Western blot with polyclonal anti-flag
antibody demonstrating flag epitope-tagged human Beclin 1
expression in JCY3000 + pRS424.*beclin1* yeast transformants.
Positive control (lane 1) represents lysate of mammalian
cells transfected with mammalian flag-*beclin 1* expression
25 vector. Negative controls (lanes 2, 3, 4) represent
SEY6210, JCY3000, and JCY3000 + pRS424. *vps30* yeast,
respectively. Lanes 5 and 6 represent independent JCY3000
yeast clones (clones *beclin 1.3* and *beclin 1.5*) with
homologous recombination replacement of flag *beclin 1* for
30 the *apg6/vps30* ORF in the plasmid pRS424.*vps30*. b,
Photomicrographs of autophagic body formation in yeast
subjected to nitrogen deprivation in the presence and
absence of 1 mM PMSF. SEY6210 is wild-type, and JCY3000 is
disrupted of *apg6/vps30*. Arrows denote representative cells
35 which would be scored as positive in experiment shown in c.

-20-

c, Quantitative effects of *apg6/vps30* and *beclin 1*
transformation   on   autophagic   body   formation   in
apg6/vps30-disrupted yeast in the presence of PMSF.
Autophagic bodies were defined as visible bodies within the
vacuole of the yeast (see arrows in **b**). A minimum of 100
cells was counted for each sample for each yeast strain.
The results shown represent the mean ± S.E.M. for triplicate
samples. Similar results were obtained in three independent
experiments.

Figure 24

Promotion of autophagy by enforced *beclin 1* expression in
human MCF7 breast carcinoma cells. Representative electron
micrographs of MCF7.control clone 38 (**a,c**) and MCF7.*beclin1*
clone 17 cells (**b, d, e**) grown in nutrient-rich media (**a,
b**) or subjected to 3.5 hours of serum and amino acid
deprivation (**c-e**). Asterisks in **e** denote representative
autophagic vacuoles which would be counted in experiment
shown in **f**. Scale bars = 2 _m. **f**, Quantitative effects
of *beclin 1* on basal and nutrient-deprivation induced
autophagy of MCF7 cells. Solid bars indicate mean ± S.E.M.
number of autophagic vacuoles per cell for cells growing in
normal media. Hatched bars indicate mean ± S.E.M. number
of autophagic vacuoles per cell for cells subjected to 3.5
hours of serum and amino acid deprivation.   Open bars
indicate mean ± S.E.M. number of autophagic vacuoles per
cell for cells pre-treated for 30 minutes with 10 mM
3-methyladenine, and then subjected to 3.5 hours of serum
and amino acid deprivation. The mean number of autophagic
vacuoles per cell was determined by counting the total
number of autophagic vacuoles in each cell for 100 cells per
clone per treatment group. Autophagic vacuoles were defined
a double-membrane containing vacuolar structures containing
recognizable   cytoplasmic   contents   (see   representative
vacuoles in **e**).

Figure 25

Effects of enforced *beclin 1* expression on growth properties
and tumorigenicity of MCF7 cells. **a,** Western blot analysis
of flag epitope-tagged *beclin 1*-transfected MCF7 clones using
5   anti-flag M2 antibody. (+) control represents lysates of BHK
cells infected with Sindbis virus vector expressing
flag-Beclin 1[2]. (-) control represents MCF7.control clone
38. **b,** Representative photomicrographs demonstrating
morphologic appearance of MCF7.control clones (left panel,
10 clone 5) and MCF7.*beclin1* clones (right panel, clone 17) 48
hours after seeding at similar densities. **c,** Proliferation
of MCF7.control clones (closed symbols) and MCF7.*beclin1*
clones (open symbols). Y axis represents O.D. values measured
by MTT assay. Results shown represent mean + S.E.M. for
15 triplicate wells. Similar results were obtained in three
independent experiments. **d,** Clonigenicity in semi-solid
medium (soft-agar) of MCF7.control and MCF7.*beclin1* clones.
Results shown represent mean ± S.E.M. for pooled triplicate
wells from 3-4 independent experiments. **e,** Tumor formation
20 in NCR nude mice injected subcutaneously with MCF.control and
MCF.*beclin1* clones. Numbers on top of bar represent number
of autopsy-confirmed tumors at eight weeks/ number of mice
injected with each clone.   Immunoperoxidase staining to
detect flag-Beclin 1 in MCF7.control clone 5 (top row) and
25 MCF.*beclin1* clone 17 cells (bottom row) prior to injection
into nude mice (left column) and in tumors arising in nude
mice (right column) using anti-flag M2 antibody. Arrow
denotes cluster of flag-Beclin 1 immunoreactive cells.

30 Figure 26

Decrease in Beclin 1 protein expression in human breast
cancer cell lines and tissue. **a,** Western blot analysis using
anti-Beclin 1 and anti-actin antibodies of representative
breast cancer cell lines. **b,** Western blot analysis using
35 anti-Beclin 1 and anti-actin antibodies of matched normal and

malignant breast tissue from representative patients with
sporadic invasive breast carcinoma.   c, Immunoperoxidase
staining using anti-Beclin 1 antibody of paraffin-embedded
sections of matched normal and malignant breast tissue from
5 two patients shown in **b**.   In **b** and **c**, N = normal breast
tissue, T = tumor tissue.

Figure 27
Western Blot analysis of Beclin 1 expression in MCF7 cells
10 treated with tamoxifen.

DETAILED DESCRIPTION OF THE INVENTION

In order to facilitate an understanding of the Experimental
Details section which follows, certain frequently occurring
methods and/or terms are described in Sambrook, et al.(1989).

Throughout this application, references to specific
nucleotides are to nucleotides present on the coding strand
of the nucleic acid. The following standard abbreviations
are used throughout the specification to indicate specific
nucleotides:

    C=cytosine   A=adenosine
    T=thymidine   G=guanosine

In an embodiment, a "gene" includes a nucleic acid molecule,
the sequence of which includes all the information required
for the normal regulated production of a particular protein,
including the structural coding sequence, promoters and
enhancers.

In an embodiment a wildtype human Beclin includes a
polypeptide which has an amino acid sequence identical to
that present in a naturally-occurring form of human Beclin.
In an embodiment, a mutant human Beclin includes a
polypeptide having an amino acid sequence which differs by
one or more amino residues from any naturally occurring form,
including deletions mutants containing less than all of the
residues present in the wildtype polypeptide, substitution
homologs wherein one or more residues are replaced by other
residues, and addition homologs wherein on or more amino acid
residues are added to a terminal or medial portion of the
polypeptide.

The nucleic acids and oligonucleotides described and claimed
herein are useful for the information which they provide
concerning the amino acid sequence of the polypeptide and as
products for the large scale synthesis of the polypeptide by
5   a variety of recombinant techniques. The molecule is useful
for   generating   new   cloning   and   expression   vectors,
transformed and transfected prokaryotic and eukaryotic host
cells, and new and useful methods for cultured growth of such
host cells capable of expression of the polypeptide and
10 related products.

The present invention provides for an isolated nucleic acid
which encodes a wildtype human Beclin. This invention further
provides an isolated nucleic acid which encodes a mutant
15 human Beclin. The above-described isolated nucleic acids can
be DNA, specifically cDNA or genomic DNA, and RNA.

In   an   embodiment,   a "mutant   human   Beclin"   includes
polypeptides that whose nucleic acid sequence or amino acid
20 seqeunce differs from that of the naturally-occuring wildtype
human Beclin.   For example, due to a point mutation, the
translated polypeptide differs from the naturally-occuring
wildtype protein. Further, a subject may have low expression
of the naturally-occurring protein so that the cells with
25 this   low-expressing   protein   cannot   inhibit   cell
proliferation.

This invention also provides for a vector comprising the
above-described nucleic acid operatively linked to a promoter
30 of RNA transcription.

Numerous vector backbones are known in the art and are useful
for expressing proteins.   Such vectors include plasmid
vectors, cosmid vectors, yeast artificial chromosome (YAC),
35 bacteriophage or eukaryotic viral DNA. For example, one such
class of vectors comprises DNA elements derived from viruses

such as bovine papilloma virus, polyoma virus, adenovirus,
vaccinia virus, baculovirus, retroviruses (RSV, MMTV or
MoMLV), Semliki Forest virus or SV40 virus. Such vectors may
be obtained commercially or assembled from the sequences
5 described by methods well-known in the art.

This invention specifically provides a plasmid designated
pSG5/beclin. Plasmid pSG5/beclin was made by cleaving DNA
which encodes a wildtype human Beclin and inserting the DNA
10 into the Eco RI site of pSG5 (Figure 7). pSG5/beclin was
deposited on July 18, 1996 with the American Type Culture
Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland
20852, U.S.A., under the provisions of the Budapest Treaty
For The International Recognition Of The Deposit Of
15 Microorganisms For The Purposes Of Patent Procedure.
pSG5/beclin has been accorded ATCC Accession Number 97664.

These vectors may be introduced into a suitable host cell to
20 form a host vector system for producing the inventive
proteins.   Methods of making host vector systems are well
known to those skilled in the art.

Suitable host cells include, but are not limited to,
25 bacterial cells (including gram positive cells), yeast cells,
fungal cells, insect cells and animal cells. Suitable animal
cells include, but are not limited to HeLa cells, Cos cells,
CV1 cells and various primary mammalian cells.   Numerous
mammalian cells may be used as hosts, including, but not
30 limited to, the mouse fibroblast cell NIH-3T3 cells, CHO
cells, HeLa cells, Ltk⁻ cells and COS cells. Mammalian cells
may be transfected by methods well known in the art such as
calcium phosphate precipitation, electroporation and
microinjection.

35

This invention also provides a host vector system for the
production of a polypeptide which comprises the above-
described vector in a suitable host.

5   This invention also provides a method of producing a
polypeptide which comprises growing the above-described host
vector system, under suitable conditions permitting
production of the polypeptide and recovering the polypeptide
so produced. Further, this invention also provides a method
10 of obtaining a polypeptide in purified form which comprises
(a) introducing the above-described vector into a suitable
host cell, (b) culturing the resulting host cell so as to
produce the polypeptide, (c) recovering the polypeptide
produced into step (b); and (d) purifying the polypeptide so
15 recovered.   In the above-described method, the vector
comprises a plasmid, cosmid, yeast artificial chromosome
(YAC), bacteriophage or eukaryotic viral DNA and the suitable
host cell comprises a bacterial, insect, plant or mammalian
cell.
20

This invention also provides a purified, wildtype human
Beclin. In an embodiment, wildtype human Beclin includes a
polypeptide which has an amino acid sequence identical to
that present in a naturally-occurring form of human Beclin.
25

This invention also provides an oligonucleotide of at least
15 nucleotides capable of specifically hybridizing with a
unique sequence of nucleotides within a nucleic acid which
encodes a wildtype Beclin without hybridizing to any sequence
30 of nucleotides within a nucleic acid which encodes a mutant
human Beclin.   Further, this invention also provides an
oligonucleotide of at least 15 nucleotides capable of
specifically hybridizing with a unique sequence of
nucleotides within a nucleic acid which encodes a mutant
35 Beclin without hybridizing to any sequence of nucleotides
within a nucleic acid which encodes a wildtype human Beclin.

The above-described oligonucleotides may DNA or RNA. Methods
of manufacturing such oligonucleotides and using the
oligonucleotides are well-known in the art.

5   This invention also provides a method for determining whether
a subject has a predisposition for cancer which comprises (a)
obtaining an appropriate nucleic acid sample from the
subject; and (b) determining whether the nucleic acid sample
from step (a) is, or is derived from, a nucleic acid which
10 encodes a mutant human Beclin so as to thereby determine
whether a subject has a predisposition for cancer. Various
methods of determining whether the nucleic acid sample is,
or is derived from, a nucleic acid which encodes a mutant
human Beclin exist.

15

In one example, the nucleic acid sample in step (a) comprises
mRNA corresponding to the transcript of DNA encoding a mutant
Beclin, and wherein the determining of step (b) comprises (i)
contacting the mRNA with the above-described oligonucleotide,
20 which is capable of specifically hybridizing with a unique
sequence of nucleotides within a nucleic acid which encodes
a mutant Beclin without hybridizing to any sequence of
nucleotides within a nucleic acid which encodes a wildtype
human Beclin, under conditions permitting binding of the mRNA
25 to the oligonucleotide so as to form a complex, (ii)
isolating the complex so formed, and (iii) identifying the
mRNA in the isolated complex so as to thereby determine
whether the mRNA is, or is derived from, a nucleic acid which
encodes a mutant human Beclin.

30

In another example, the determining of step (b) comprises (i)
contacting the nucleic acid sample of step (a), and the
isolated nucleic acid which encodes a wildtype human Beclin
with restriction enzymes under conditions permitting the
35 digestion of the nucleic acid sample, and the isolated
nucleic acid into distinct, distinguishable pieces of nucleic

acid, (ii) isolating the pieces of nucleic acid; and (iii)
comparing the pieces of nucleic acid derived from the nucleic
acid sample with the pieces of nucleic acid derived from the
isolated nucleic acid so as to thereby determine whether the
5   nucleic acid sample is, or is derived from, a nucleic acid
which encodes a mutant human Beclin.

In another example, the determining of step (b) comprises (i)
sequencing the nucleic acid sample of step (a); and (ii)
10   comparing the nucleic acid sequence of step (i) with the
above-described isolated nucleic acid having a sequence
substantially the same as the nucleotide sequence of figure
1B, so as to thereby determine whether the nucleic acid
sample is, or is derived from, a nucleic acid which encodes
15   a mutant human Beclin.

In another example, the nucleic acid sample in step (a)
comprises mRNA corresponding to the transcript of DNA
encoding mutant Beclin, and wherein the determining of step
20   (b) comprises (i) translating the mRNA under suitable
conditions to obtain an amino acid sequence; and (ii)
comparing the amino acid sequence of step (i) with the above-
described isolated nucleic acid which has the amino acid
sequence shown in figure 1A so as to thereby determine
25   whether the nucleic acid sample is, or is derived from, a
nucleic acid which encodes a mutant human Beclin.

In another example, the determining of step (b) comprises (i)
amplifying the nucleic acid present in the sample of step
30   (a); and (ii) detecting the presence of the mutant human
Beclin in the resulting amplified nucleic acid.

The above-described methods of determining are well-known to
those skilled in the art. In an embodiment, the isolated
35   nucleic acid or the oligonucleotide is labeled with a

detectable marker, wherein the detectable marker is a radioactive isotope, a fluorophor or an enzyme.

Further, in a specific embodiment, the sample comprises
5 blood, tissue or sera.

Further, the cancer includes, but is not limited to, ovarian, breast or prostrate cancers.

10 This invention also provides a method for determining whether a subject has cancer, which comprises (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes a mutant
15 human Beclin so as to thereby determine whether a subject has cancer.

Various methods of determining whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes
20 a mutant human Beclin exist.

In one example, the nucleic acid sample in step (a) comprises mRNA corresponding to the transcript of DNA encoding a mutant Beclin, and wherein the determining of step (b) comprises (i)
25 contacting the mRNA with the above-described oligonucleotide, which is capable of specifically hybridizing with a unique sequence of nucleotides within a nucleic acid which encodes a mutant Beclin without hybridizing to any sequence of nucleotides within a nucleic acid which encodes a wildtype
30 human Beclin, under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex, (ii) isolating the complex so formed, and (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which
35 encodes a mutant human Beclin.

In another example, the determining of step (b) comprises (i)
contacting the nucleic acid sample of step (a), and the
isolated nucleic acid which encodes a wildtype human Beclin
with restriction enzymes under conditions permitting the
5   digestion of the nucleic acid sample, and the isolated
nucleic acid into distinct, distinguishable pieces of nucleic
acid, (ii) isolating the pieces of nucleic acid; and (iii)
comparing the pieces of nucleic acid derived from the nucleic
acid sample with the pieces of nucleic acid derived from the
10 isolated nucleic acid so as to thereby determine whether the
nucleic acid sample is, or is derived from, a nucleic acid
which encodes a mutant human Beclin.

In another example, the determining of step (b) comprises (i)
15 sequencing the nucleic acid sample of step (a); and (ii)
comparing the nucleic acid sequence of step (i) with the
above-described isolated nucleic acid having a sequence
substantially the same as the nucleic acid sequence shown in
figure 1B, so as to thereby determine whether the nucleic
20 acid sample is, or is derived from, a nucleic acid which
encodes a mutant human Beclin.

In another example, the nucleic acid sample in step (a)
comprises mRNA corresponding to the transcript of DNA
25 encoding mutant Beclin, and wherein the determining of step
(b) comprises (i) translating the mRNA under suitable
conditions to obtain an amino acid sequence; and (ii)
comparing the amino acid sequence of step (i) with the above-
described isolated nucleic acid which has the amino acid
30 sequence shown in figure 1A so as to thereby determine
whether the nucleic acid sample is, or is derived from, a
nucleic acid which encodes a mutant human Beclin.

In another example, the determining of step (b) comprises (i)
35 amplifying the nucleic acid present in the sample of step

(a),   and (ii) detecting the presence of the mutant human
Beclin in the resulting amplified nucleic acid.

The above-described methods of determining are well-known to
5 those skilled in the art.   In an embodiment, the isolated
nucleic acid or the oligonucleotide is labeled with a
detectable marker, wherein the detectable marker is a
radioactive isotope, a fluorophor or an enzyme.

10 Further, in a specific embodiment, the sample comprises
blood, tissue or sera.

Further, the cancer includes, but is not limited to, ovarian
or breast cancers.
15

This invention also provides for a method for inhibiting cell
proliferation in cells, suppressing tumor activity   or
inducing autophagy by introducing the isolated nucleic acid
which encodes a wildtype human Beclin into the cells,
20 specifically wherein the cells are cancerous. Various methods
of introducing nucleic acids into cells are well-known to
those skilled in the art.

This invention also provides a method for treating a subject
25 who has cancer which comprises introducing the isolated
nucleic acid which encodes a wildtype human Beclin, into the
subject so as to thereby treat the cancer. Various methods
of introducing nucleic acids into cells are well-kwown to
those skilled in the art. In one example, one can introduce
30 the isolated nucleic acid by (a) recovering cancer cells from
the subject, (b) introducing the isolated nucleic acid of
claim 1 into the cells; and (c) reintroducing the cells of
step (b) into the subject so as to treat the subject who has
cancer. Many types of cancer cells exist and are well-known
35 in the art, specifically, breast, ovarian, skeletal,
cervical, colon, prostate or lung cells.

This invention also provides a pharmaceutical composition comprising a purified wildtype human Beclin and a pharmaceutically acceptable carrier. This invention further provides a pharmaceutical composition comprising the

5 polypeptide obtained from using the above-described method of obtaining a polypeptide in a purified form and a pharmaceutically acceptable carrier.

This invention also provides a method for treating a subject

10 who has cancer comprising administration of an effective amount of the above-described pharmaceutical compositions to the subject who has cancer. The administration of the pharmaceutical compositions may be by topical, oral, aerosol, subcutaneous administration, infusion, intralesional,

15 intramuscular, intraperitoneal, intratumoral, intratracheal, intravenous injection, or liposome-mediate delivery.

This invention also provides a method for detecting the presence of human chromosomal region 17q21 in a sample of

20 genomic DNA which comprises (a) contacting the sample with the isolated nucleic acid which encodes a wildtype human Beclin, under conditions permitting formation of a complex between any genomic DNA present in the sample that is complementary to such nucleic acid, and (b) detecting the

25 presence of any complex formed in step (a), the presence of such a complex indicating the human chromosomal region 17q21 is present in the sample. Further, one may contacting the sample with an oligonucleotide, which is capable of specifically hybridizing with a unique sequence of

30 nucleotides within a nucleic acid which encodes a mutant Beclin without hybridizing to any sequence of nucleotides within a nucleic acid which encodes a wildtype human Beclin, under conditions permitting formation of a complex between any genomic DNA present in the sample that is complementary

35 to such oligonucleotide, and (b) detecting the presence of any complex formed in step (a), the presence of such a

complex indicating the human chromosomal region 17q21 is
present in the sample. The nucleic acid may be labeled with
a detectable marker, wherein the detectable marker is a
radioactive isotope, a fluophor or an enzyme.

5

This invention also provides a method for detecting a mutant
human Beclin in a subject which comprises (a) obtaining an
appropriate nucleic acid sample from the subject, and (b)
determining whether the nucleic acid sample from step (a) is,
10 or is derived from, a nucleic acid which encodes mutant human
Beclin so as to thereby detect a mutant human Beclin in the
subject.

Various methods of determining whether the nucleic acid
15 sample is, or is derived from, a nucleic acid which encodes
a mutant human Beclin exist.

In one example, the nucleic acid sample in step (a) comprises
mRNA corresponding to the transcript of DNA encoding a mutant
20 Beclin, and wherein the determining of step (b) comprises (i)
contacting the mRNA with the above-described oligonucleotide,
which is capable of specifically hybridizing with a unique
sequence of nucleotides within a nucleic acid which encodes
a mutant Beclin without hybridizing to any sequence of
25 nucleotides within a nucleic acid which encodes a wildtype
human Beclin, under conditions permitting binding of the mRNA
to the oligonucleotide so as to form a complex, (ii)
isolating the complex so formed, and (iii) identifying the
mRNA in the isolated complex so as to thereby determine
30 whether the mRNA is, or is derived from, a nucleic acid which
encodes a mutant human Beclin.

In another example, the determining of step (b) comprises (i)
contacting the nucleic acid sample of step (a), and the
35 isolated nucleic acid which encodes a wildtype human Beclin
with restriction enzymes under conditions permitting the

digestion of the nucleic acid sample, and the isolated
nucleic acid into distinct, distinguishable pieces of nucleic
acid, (ii) isolating the pieces of nucleic acid; and (iii)
comparing the pieces of nucleic acid derived from the nucleic
5   acid sample with the pieces of nucleic acid derived from the
isolated nucleic acid so as to thereby determine whether the
nucleic acid sample is, or is derived from, a nucleic acid
which encodes a mutant human Beclin.

10   In another example, the determining of step (b) comprises (i)
sequencing the nucleic acid sample of step (a); and (ii)
comparing the nucleic acid sequence of step (i) with   the
above-described isolated nucleic acid having a sequence
substantially the same as the nucleic acid sequence shown in
15   figure 1B, so as to thereby determine whether the nucleic
acid sample is, or is derived from, a nucleic acid which
encodes a mutant human Beclin.

In another example, the nucleic acid sample in step (a)
20   comprises mRNA corresponding to the transcript of DNA
encoding mutant Beclin, and wherein the determining of step
(b) comprises (i) translating the mRNA under suitable
conditions to obtain an amino acid sequence; and (ii)
comparing the amino acid sequence of invention also provides
25   a method for treating a subject unable to control step (i)
with the above-described isolated nucleic acid which has the
amino acid sequence shown in figure 1A so as to thereby
determine whether the nucleic acid sample is, or is derived
from, a nucleic acid which encodes a mutant human Beclin.
30
In another example, the determining of step (b) comprises (i)
amplifying the nucleic acid present in the sample of step
(a); and (ii) detecting the presence of the mutant human
Beclin in the resulting amplified nucleic acid.
35

The above-described methods of determining are well-known to
those skilled in the art. In an embodiment, the isolated
nucleic acid or the oligonucleotide is labeled with a
detectable marker, wherein the detectable marker is a
5   radioactive isotope, a fluorophor or an enzyme.

Further, in a specific embodiment, the sample comprises
blood, tissue or sera.

10 This apoptosis in the cells of the subject which comprises
introducing the isolated nucleic acid of figure 1, into the
subject so as to treat the subject unable to control
apoptosis in the cells of the subject. In a specific
embodiment, the cells are cancerous. Various method of
15 introducing isolated nucleic acids into cells exist and are
well-known in the art. In one example, one can introduce the
isolated nucleic acid by (a) recovering cancer cells from the
subject, (b) introducing the isolated nucleic acid of claim
1 into the cells; and (c) reintroducing the cells of step (b)
20 into the subject so as to treat the subject who has cancer.

This invention also provides a method of treating a subject
unable to control apoptosis in the cells of the subject
comprising administration of an effective amount of the
25 above-described pharmaceutical compositions to the subject,
wherein the administration comprises topical, oral, aerosol,
subcutaneous administration, infusion, intralesional,
intramuscular, intraperitoneal, intratumoral, intratracheal,
intravenous injection, or liposome-mediate delivery.
30 This invention provides a method for treating a subject so
as to restore cell growth control in the cells of the subject
which comprises introducing the isolated nucleic acid of of
figure 1, into the subject so as to treat the subject unable
to control cell growth in the cells of the subject. In a
35 specific embodiment, the cells are cancerous. Various method
of introducing isolated nucleic acids into cells exist and

are well-known in the art. In one example, one can introduce
the isolated nucleic acid by (a) recovering cancer cells from
the subject, (b) introducing the isolated nucleic acid of
claim 1 into the cells; and (c) reintroducing the cells of
5   step (b) into the subject so as to treat the subject who has
cancer.

This invention provides a method for inhibiting viral
replication in a subject comprising contacting an effective
10 amount of Beclin with the virus infected cell, thereby
inhibiting the viral replication or by inducing the
expression of Beclin within the virus infected cell thereby
inhibiting the viral replication.

15 This invention provides a method for treating a viral
infection in a subject, wherein the viral infection is
alleviated by the expression of Beclin comprising
administration of an effective amount of a pharmaceutical
composition effective to express Beclin, wherein the
20 administering of beclin comprises (a) recovering infected
cells from the subject (b) introducing the isolated beclin
into the cells and (c) reintroducing the cells of step (b)
into a subject sos as to treat the infected subject.

25 In the practice of any of the methods of the invention or
preparation of any of the pharmaceutical compositions an
"therapeutically effective amount" is an amount which is
capable of inhibiting hairlessness or T-cell deficiency.
Accordingly, the effective amount will vary with the subject
30 being treated, as well as the condition to be treated. For
the purposes of this invention, the methods of administration
are to include, but are not limited to, administration
cutaneously, subcutaneously, intravenously, parenterally,
orally, topically, or by aerosol.
35

As used herein, the term "suitable pharmaceutically
acceptable carrier" encompasses any of the standard
pharmaceutically accepted carriers, such as phosphate
buffered saline solution, water, emulsions such as an
5 oil/water emulsion or a triglyceride emulsion, various types
of wetting agents, tablets, coated tablets and capsules. An
example of an acceptable triglyceride emulsion useful in
intravenous and intraperitoneal administration of the
compounds is the triglyceride emulsion commercially known as
10 Intralipid®.

Typically such carriers contain excipients such as starch,
milk, sugar, certain types of clay, gelatin, stearic acid,
talc, vegetable fats or oils, gums, glycols, or other known
15 excipients. Such carriers may also include flavor and color
additives or other ingredients.

This invention also provides for pharmaceutical compositions
capable of inhibiting neurotoxicity together with suitable
20 diluents, preservatives, solubilizers, emulsifiers, adjuvants
and/or carriers. Such compositions are liquids or
lyophilized or otherwise dried formulations and include
diluents of various buffer content (e.g., Tris-HCl., acetate,
phosphate), pH and ionic strength, additives such as albumin
25 or gelatin to prevent absorption to surfaces, detergents
(e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts),
solubilizing agents (e.g., glycerol, polyethylene glycerol),
anti-oxidants (e.g., ascorbic acid, sodium metabisulfite),
preservatives (e.g., Thimerosal, benzyl alcohol, parabens),
30 bulking substances or tonicity modifiers (e.g., lactose,
mannitol), covalent attachment of polymers such as
polyethylene glycol to the compound, complexation with metal
ions, or incorporation of the compound into or onto
particulate preparations of polymeric compounds such as
35 polylactic acid, polglycolic acid, hydrogels, etc., or onto
liposomes, micro emulsions, micelles, unilamellar or multi
lamellar vesicles, erythrocyte ghosts, or spheroplasts. Such
compositions will influence the physical state, solubility,

stability, rate of *in vivo* release, and rate of *in vivo* clearance of the compound or composition.

Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

When administered, compounds are often cleared rapidly from the circulation and may therefore elicit relatively short-lived pharmacological activity. Consequently, frequent injections of relatively large doses of bioactive compounds may by required to sustain therapeutic efficacy. Compounds modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline are known to exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified compounds (Abuchowski et al., 1981; Newmark et al., 1982; and Katre et al., 1987). Such modifications may also increase the compound's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the compound, and greatly reduce the immunogenicity and reactivity of the compound. As a result, the desired *in vivo* biological activity may be achieved by the administration of such polymer-compound adducts less frequently or in lower doses than with the unmodified compound.

Further, in a specific embodiment, the virus comprises an RNA
virus, a Sindbis virus, a mutated Sindbis virus or an
influenza virus.

5   This invention provides a method of diagnosing a
    predisposition to carcinoma in a subject comprising: a)
    obtaining a nucleic acid sample from the subject; and b)
    determining whether one or more of the subject's beclin
    alleles or regulatory regions to those alleles are deleted
10 or different from the wild type so as to reduce the subject's
    expression of polypeptide having tumor suppressor activity.

    In an embodiment, a predisposition to carcinoma in a subject
    means that the subject based upon the diagnostic result(s)
15 has a greater likelihood of developing a carcinoma than an
    otherwise similar subject lacking the diagnostic result. In
    an embodiment, a predisposition to carcinoma in a subject
    means that the subject based upon the diagnostic result(s)
    has a greater likelihood of developing carcinoma of greater
20 severity than an otherwise similar subject lacking the
    diagnostic result. One skilled in the art would recognize
    the statistical nature of diagnosing a predisposition to
    carcinoma. In an embodiment, Bayes Theorem is applied to
    assessing the increased likelihood of carcinoma.

25

    Beclin allele and regulatory element differences from the
    wild type include, but are not limited to, deletions,
    additions, substitutions, and chemical modification of
    beclin. Chemical modifications of beclin and/or its
30 regulatory elements include, but are not limited to,
    nucleotide phosphorylation, methylation, and/or
    hydroxylation. Sites of nucleotide differences include, but
    are not limited to, introns, exons, enhancers, promoters,
    splice junctions, splice site consensus sequences, RNA-
35 cleavage/polyadenylation sites, cap sites, and other protein
    binding sites. In an embodiment, protein binding sites are

located by "footprinting", wherein a protein shields the
nucleic acid from cleavage by restriction enzymes.

Beclin allele or regulatory differences also include, but are
5   not limited to, sequence changes that alter the relative
distance of the beclin allele from its regulatory elements.
In an embodiment, an insertion increases the distance of
beclin from an enhancer. In another embodiment, an deletion,
increase the proximity of beclin to its promoter.

10

Regulatory regions include, but are not limited to, promoters
and enhancers.

This invention also provides the above-described method,
15   wherein one or more beclin alleles contain splice site
junction mutations.

This invention further provides the above-described methods,
wherein there are one or more mutations in the 100 base pair
20   region centered about the splice acceptor site consensus
sequence located upstream of the exon spanning the
nucleotides corresponding to positions 1042-1185 of figure
1B or one or more mutations in the 100 base pair region
centered about the splice donor site consensus sequence
25   located downstream of the exon spanning the nucleotides
corresponding to positions 1042-1185 of figure 1B.

Splice site junction mutations include but are not limited
to deletions, additions, and/or substitutions at or near the
30   splice site consensus sequence. In an embodiment, the splice
site consensus sequence overlaps both the adjoining intron
and exon.   In an embodiment, the mutations at or near the
splice site consensus sequence reduce the recognition of the
splice site by splicing factors. One skilled in the art
35   would recognize that mutations in splice site consensus
sequences can reduce or eliminate splicing at these

locations. Further, one skilled in the art would recognize
that if the wild type splice site has been mutated, the other
sites may become used as splice sites with increased
frequency. Further, one skilled in the art would also
5 recognize that mutations can result in the creation of new
splice sites. One skilled in the art would be able to
determine from the mRNA or cDNA thereto whether the mRNA or
cDNA is a product of aberrant splicing.

10 Primers were used to amplify exon-intron boundaries
corresponding to regions of possible splice site mutations
in ovarian cancer. Aberrant *beclin* cDNAs have been
identified in ovarian tumors indicative of splice site
mutations upstream or downstream of the exon spanning from
15 1082-1185. To facilitate identification of the genomic DNA
beclin splice site mutations responsible for these mutant
transcripts, two sets of primers for use in PCR amplification
of relevant exon-intron boundaries were designed. These two
sets of primers are:
20
1.   Analysis of exon-intron boundaries for 1041/1042
        splice site junctions:
        Foward primer (nucleotides 984-1003) of beclin)
        CCGACTTGTTCCTTACGGAA
25   Reverse primer (nucleotides 1166-1147 of beclin)
        CGTGTCTCGCCTTTCTCAAC
2.   Analysis of exon/intron boundaries for 1185/1186
        splice site junction:
        Forward primer (nucleotides 1070-1089 of beclin)
30   TGCGGTTTTTCTGGGACAAC
        Reverse primer (nucleotides 1325-1306 of beclin)
        CAAGCAAGACCCCACTTAAG

In addition, this invention provides the above-described
35 methods, wherein the determining of step b comprises

determining whether one or more beclin promoter regions
differ from the wild type.

One skilled in the art would recognize that mutations in
5 beclin promoter regions may result in reduced expression of
Beclin and thus reduced tumor suppressor function. Such
changes would lead to a predisposition to carcinoma.

This invention provides also the above-described methods,
10 wherein the determining of step b comprises determining
whether one or more beclin promoter regions are
hypermethylated.

In an embodiment, hypermethylation is associated with regions
15 of DNA that have reduced activity or no activity. In an
embodiment methylation of beclin promoter sequences is likely
to reduce transciption of beclin. In an embodiment,
methylation of other regions of beclin, besides the promoter,
including the coding region would also be indicative of
20 reduced Beclin activity. In an embodiment methylation of
beclin or its regulatory regions occurs at CG pairs. In
another embodiment, methylation of beclin or its regulatory
regions occurs at sites containing a high a frequency of CG
pairs.
25

In an embodiment, methylation is assessed by using
restriction endonucleases whose recognition sequences contain
CG. For example, HpaII and MspI both recognize CCGG.
However, HspII cleaves only unmethylated DNA while MspI
30 cleaves both methylated and unmethylated DNA. Using these
two restriction enzymes, one skilled in the art could infer
from the size of the restriction fragments the presence of
methylation in beclin or its regulatory elements.

35 Further, this invention provides the above-described methods,
wherein the determining of step b comprises determining

whether one or more beclin enhancer regions differ from the
wild type.

In an embodiment, enhancer regions may be located upstream
5   or downstream of Beclin. In an embodiment, mutations in
beclin enhancer regions would reduce Beclin production and
thus lead to an increased predisposition for carcinoma.

This invention further provides the above-described methods,
10 wherein the determining of step b comprises determining
whether one or more beclin 5' or 3' nontranslated regions
differ from the wild type.

In an embodiment, nontranslated regions are responsible for
15 regulating the levels of gene activity. In an embodiment,
mutations in these regions would be expected to reduce Beclin
production and thus lead to an increased predisposition for
carcinoma.

20 This invention also provides the above-described methods,
wherein the determining of step b comprises determining
whether one or more beclin alleles have a premature
termination codon.

25 In addition, this invention provides the above-described
methods, wherein the determining of step b comprises
determining whether there is a premature termination codon
in the nucleic acid corresponding to positions 1180-1240 of
figure 1B.
30
In an embodiment, alleles having a premature termination
codon would lead to truncated products having no tumor
suppressor activity or reduced tumor suppressor activity.
In another embodiment, a premature termination codon between
35 positions 1180-1240 of figure 1B would be expected to affect

a region of Beclin that is important for its tumor suppressor
functions.

This invention also provides the above-described methods,
5 wherein the determining of step b comprises contacting the
nucleic acid sample with a primer that specifically
hybridizes to the junction between the exon corresponding to
positions 1042-1185 of figure 1B and the exon adjoining
downstream thereto.

10

This invention also provides the above-described methods,
wherein the determining of step b comprises contacting the
nucleic acid sample with a primer that specifically
hybridizes to the junction between the exon corresponding to
15 positions 1042-1185 of figure 1B and the exon adjoining
upstream thereto.

In addition, this invention provides the above-described
methods, wherein the determining of step b comprises
20 contacting the nucleic acid sample with a primer that
specifically hybridizes to the junction between the exon
corresponding to positions 1042-1185 of figure 1B and the
intron adjoining downstream thereto.

25 This invention further provides the above-described methods,
wherein the determining of step b comprises contacting the
nucleic acid sample with a primer that specifically
hybridizes to the junction between the exon corresponding to
positions 1042-1185 of figure 1B and the intron adjoining
30 upstream thereto.

In an embodiment, a primer that specifically hybridizes to
the junction between the exon corresponding to positions
1042-1185 of figure 1B and the adjoining downstream or
35 upstream exon would exhibit reduced hybridization if there

was a mutation in this region. Such reduced hybridization
would be diagnostic of a splice site mutation in this region.

5   In an embodiment, a primer that specifically hybridizes to
the junction between the exon corresponding to positions
1042-1185 of figure 1B and the adjoining downstream or
upstream intron would exhibit reduced hybridization if there
was a mutation in this region. Such reduced hybridization
would be diagnostic of a splice site mutation in this region.
10

This invention also provides the above-described methods,
wherein a majority of the nucleotides corresponding to
positions 1042-1185 of figure 1B have been deleted.

15 Further, this invention provides the above-described methods,
wherein the subject was preselected based upon an abnormality
at chromosome position 17q21.

This invention also provides the above-described methods,
20 wherein the abnormality at chromosome position 17q21
comprises a deletion.

Abnormalities at chromosome position 17q21 may be detected
by numerous methodologies which include, but are not limited
25 to, karyotyping and in situ hybridization.

This invention also provides the above-described methods,
wherein the nucleic acid sample is obtained from the
subject's blood.
30

Nucleic acid samples can be obtained from numerous sources
which include, but are not limited to, blood, saliva, tissue,
and hair follicles.

35 This invention further provides a method of diagnosing a
predisposition to carcinoma in a subject, comprising

determining whether the subject has an abnormal subcellular
distribution of Beclin.

This invention also provides the above-described methods,
5 wherein the abnormal subcellular distribution of Beclin
comprises Beclin being distributed diffusely in both the
nucleus and cytoplasm.

In addition, this invention provides a method of diagnosing
10 a predisposition to carcinoma in a subject, comprising
contacting the contents of one or more of the subject's cells
with one or more antibodies or fragments thereof specific to
Beclin.

15 Further, this invention provides the above-described methods,
wherein one or more of the antibodies is the antibody with
designation number 843 and one or more of the antibodies is
the antibody with designation number 844.

20 This invention also provides the above-described methods,
wherein the antibody's failure to bind to Beclin indicates
that the subject has a predisposition to carcinoma.

In an embodiment, the antibodies are monoclonal. In another
25 embodiment, the antibodies are polyclonal. On skilled in the
art would also recognize that fragments of antibodies are
suitable for binding to antigen. In yet another embodiment,
the antibodies are labeled. Methods of labeling antibodies
include, but are not limited to, fluorescence, radioactivity,
30 and enzymatic assays. In an embodiment, the antibody is
linked to an enzyme that catalyzes a reaction leading
directly or indirectly to a detectable product.

In an embodiment, Western blotting using an antibody to
35 Beclin is used to detect Beclin. In an embodiment, the
failure of the antibody to bind to Beclin indicates that

Beclin is absent. In another embodiment, the failure of the
antibody to bind to Beclin indicates that Beclin is truncated
or altered at the region recognized by the antibody. In a
further embodiment, two or more antibodies are used that

5   recognize different epitopes of Beclin so as to distinguish
between the truncation or alteration of Beclin at or near an
epitope and the absence of Beclin. In an embodiment, the
complete failure of Beclin to be detected by Western blotting
is diagnostic of an increased risk of carcinoma.

10

Two synthetic peptides were synthesized corresponding to
amino acids 1-15 of human Beclin (MEGSKTSNNSTMQV) and amino
acids 374-389 of human Beclin (CVQQFKEEVEKGETRF). Polyclonal
antibodies to these two peptides, named 843 and 844 were

15 produced by rabbit immunizations with peptide linked to KLH
carrier.   843 exhibits strong immunoreactivity with the
Beclin peptide amino acids 1-15 in ELISA and 844 demonstrates
strong immunoreactivity with the Beclin peptide amino acids
374-389. In addition, 843 reacts with full-length wild-type

20 and mutant Beclin proteins by Western blot analysis and by
immunohistochemical staining of normal and malignant human
breast and ovarian tissues. To date Beclin immunoreactivity
using 843 has been demonstrated to be present in normal,
breat epithelial cells, normal ovarian epithelial cells,

25 vascular smooth muscle cells, skeletal muscle cells, and
neurons. Approximately 50% of sporadic invasive breast and
ovarian cancer cases have tumor epithelial cell staining for
Beclin with 843 and approximately half of these cases have
no tumor epithelial staining with 843.   Studies are in

30 progress to determine whether 844 also reacts with Beclin
protein in Western blot and immunohistochemical analyses.

843 is useful to detect total Beclin protein expression in
human cells and human tissue by Western blot or

35 immunocytochemical analysis. Because loss of Beclin protein
expression is likely to be important in breast, ovarian, and

prostate tumorigenesis, this antibody would be useful to
detect susceptibility to these cancers.

844 would be useful to distinguish normal Beclin protein
expression from mutant Beclin protein expression where the
mutant Beclin protein contains a deletion of amino acids 374-
389. Such mutant Beclin proteins are predicted to occur in
ovarian cancer, on the basis of translation of mutant cDNA
beclin sequences found in these tumors. Therefore, it is
predicted that antibody 844 cannot react with mutant Beclin
proteins made from mutant cDNA sequences found in ovarian
cancer cases. Thus, the presence of Beclin protein that
reacts with 843 but not 844 by either Western blot or
immunohistochemical analysis would be diagnostic of the
presence of a mutant Beclin protein lacking amino acids 374-
389 of wild-type Beclin protein.

In embodiments, this invention provides the above-described
methods, wherein the carcinoma is breast cancer, ovary
cancer, and/or prostate cancer. Other carcinomas may also
be diagnosed including, but not limited to, skin, colon,
rectal, lung, breast, stomach, liver, uterine, bladder, and
prostate cancers.

This invention is illustrated in the Experimental Details
section which follows. This section is set forth to aid in
understanding the invention but is not intended to, and
should not be construed to, limit in any way the invention
as set forth in the claims which follow thereafter.

EXPERIMENTAL DETAILS

FIRST SERIES OF EXPERIMENTS

5   Cell proliferation and apoptosis may share common pathways.
    Yet, in death repressor pathways, no direct links have been
    identified between cellular genes that inhibit apoptosis and
    cellular genes that inhibit proliferation. To investigate
    the mechanism by which anti-apoptotic genes function, the
10 yeast two hybrid system was used to screen an adult mouse
    brain cDNA library for genes encoding proteins that interact
    with Bcl-2. Both Bcl-2 and its related family member, Bcl-x$_L$,
    interact with a novel 60 kd protein, Beclin, encoded by a
    gene on a specific region of chromosome 17q21 that is
15 postulated to contain a tumor suppressor gene important in
    sporadic breast and ovarian cancer.   Loss of function
    mutations in the conserved BH1 domains of Bcl-2 and Bcl-x$_L$
    disrupt binding with Beclin, and antisense beclin blocks the
    ability of bcl-2 to protect cells from Sindbis virus-induced
20 apoptosis.   Furthermore, beclin overexpression inhibits the
    proliferation of rat prostate adenocarcinoma AT3 cells and
    mouse NIH 3T3 fibroblast cells as well as the formation of
    human MCF7 breast tumor in nude mice. Together, these finds
    suggest that beclin is a novel bcl-2-interacting, candidate
25 tumor suppressor gene that may link regulation of cellular
    proliferation with death repressor pathways.

    The common deletion unit, located approximately 60 kb
    centromeric to BRCA1 that is postulated to contain an
30 additional tumor suppressor gene important for ovarian and
    possibly breast cancer, contains 12 previously identified
    genes (Friedman, et al., 1995).   Six of them are known genes
    or human homologs of other species, gamma tubulin, homolog
    of *D. melanogaster* enhancer of zeste, pseudogene of HMG17,
35 homolog of Pacific electric ray VAT1, glucose-6phosphatase

and Ki antigen. The remaining six genes are novel genes, one
of which is the gene referred to as *beclin* that is described
in this invention.

5   The mapping of *beclin* to this common deletion unit on
chromosome 17q21, coupled with data that Beclin interacts
with Bcl-2 and has anti-proliferative effects, raises the
possibility that Beclin may function as a tumor suppressor
gene important in ovarian and breast cancer.

10   **Example 1:**

**Yeast Two Hybrid cDNA Library Screen To Isolate Bcl-2-
interacting Proteins.** To further understand the mechanism
15 by which *bcl-2* protects against apoptosis, the yeast two
hybrid system was used to screen a mouse brain library for
complementary cDNAs encoding proteins that bind to Bcl-2.
A bait plasmid (pGBT9/*bcl-2*) was constructed by fusing human
bcl-2 (lacking the C' terminal signal-anchor sequence to
20 ensure translocation to the nucleus) to the GAL4 DNA-binding
domain, which was cotransformed with an oligo(dT) and random
hexamer primed adult mouse brain cDNA fusion library in a
GAL4-activating domain vector, pGAD10.   pGBT9/*bcl-2* was co-
transformed with 1 x 10⁶ cDNAs from a mouse brain library
25 fused to a GAL-4 activation domain vector (Clontech), plated
onto SD medium lacking tryptophan and leucine, and after
incubation at 30°C for 4 days, screened for LacZ activity
using a colony lift filter assay. Putative interacting
clones were isolated by manipulation in leuB E. Coli, and
30 further tested against pGBT9 and control plasmids. Of one
million transformants, one true positive colony (F1) was
identified by the X-Gal filter assay. A positive β-gal
reaction between pGBT9/*bcl-2* and clone F1 was obtained within
15-20 minutes. The sequence of the Eco RI insert in clone
35 F1 was obtained using Sequenase and by automated dideoxy

sequencing. Sequencing analysis of the cDNA plasmid rescued
from this colony revealed a termination codon 42 base pairs
downstream from the GAL 4 activation domain, several
predicted short open reading frames between nucleotides 124
5 and 1843, and a longer predicted open reading frame spanning
from nucleotide 1855 to 2500(the 3' end of the insert),
suggesting that either the 14 amino acid fusion protein was
interacting with Bcl-2, or one of the downstream open reading
frames encoded a protein that contains its own activation
10 domain and interacts with Bcl-2. To identify the
Bcl-2-interacting region of F1, nucleotides 1-1854 and
1855-2500 were fused to the GAL4 activation domain in pGAD424
and tested for interactions with Bcl-2. Nucleotides
1855-2500, but not 1-1800, encoded a protein that
15 specifically interacts with Bcl-2 (Table 1) but not with
control GAL4 DNA binding domain plasmids containing p53,
lamin (Table 1) or Sindbis virus glycoproteins.

A database search revealed that the sequence of F1:1855-2500
20 overlapped with several clones isolated from a normalized
infant human brain cDNA library in the Merck EST database as
well as clones from human breast (GT197) (Rommens, 1995) and
human fibroblast cells (B32) (Friedman, 1994). Clones GT197
and B32 were both isolated in the generation of transcription
25 maps of the breast cancer susceptibility locus on chromosome
17q21 and are mapped to a region located approximately 100
kilobases centromeric to the gene *BRCA1*. They lie within a
400 kb deletion unit mapped in sporadic ovarian cancers that
contains 12 genes, including six genes with known function
30 and six novel genes (Tangir, J., et al., 1996). This minimal
deletion unit centromeric to BRCA1 is postulated to contain
a tumor suppressor gene important for sporadic breast and
ovarian cancer. These clones contain only partial open
reading frames of a novel gene that encodes a protein with
35 coiled coils. The gene was assigned the name *beclin*, because

of the interaction of its encoded protein with bcl-2 (*bcl*) and the predicted coiled coil structure of its encoded protein (*in* suffix).

5   The overlapping partial clones in Genbank were aligned with the mouse *beclin* sequence to obtain a predicted sequence of the full-length open reading frame for human *beclin*. Human *beclin* was isolated from a normalized human brain infant cDNA library (Soares, 1994). Human Beclin is homologous with the
10 *C. elegans* T19E7.3 gene product (GENBANK accession U42843) and the *S. cerevisiae* gene product Lph7p (GENBANK accession U43503) (38% and 37% identical over 145 and 137 residues, respectively), indicating a high degree of evolutionary conservation.

Table 1.   Summary of yeast two-hybrid assay results

GAL4 AD	GAL4 BD						
	Empty	Bcl-2	Bcl-X_L	Bcl-X_s	Bax	Lamin	p53
Empty	-	-	-	+	-	-	-
F1	-	+	+	ND	-	-	-
F1:1-1855	-	-	-	ND	-	-	-
F1:1856-2563 (Mus Beclin 1-708)	-	+	+	ND	-	-	-
Hu Beclin 1-708	-	+	+	ND	-	-	-
Hu Beclin 1-450	-	+	+	ND	-	-	-
Hu Beclin 1-258	-	-	-	ND	-	-	-
Hu Beclin 262-450	-	+	+	ND	-	-	-
Hu Beclin 451-708	-	-	-	ND	-	-	-
Hu Beclin 1-1353	-	-	-	ND	-	-	-

The sequences encoding amino acids 1-218 of human bcl-2, 1-212 of bcl-x_L,
1-149 of bcl-x_S, and 1-171 of bax were cloned into pGBT9 in frame with
the GAL4-binding domain. To avoid problems with targeting or proteins
to the nucleus, the sequence encoding C' terminal transmembrane domains
were omitted. To construct pGBT9/bcl-2, human bcl-2 was amplified by PCR
from the plasmid pZIP/bcl-2, subcloned into pCR^{MII}, and the correct
sequence of bcl-2 was confirmed prior to cloning an EcoR I-Sal I fragment
into pGBt9. To construct pGBT9/bcl-x_L, pGBT9/bcl-x_s, and pGBT9/bax, the
EcoR I- Xho I fragment were excised from pGEG202 plasmids previously
described and cloned into the Eco RI - Sal I sites of pGBT9. Control
pB=GBT9 plasmids containing lamin (pLAM 5') and p53 (ppVA3) inserts were
obtained from Clontech. pGBT9/bcl-2 was co-transformed with 1 x 10⁶
cDNAs from a mouse brain library fused to a GAL-4 activation domain
vector (Clontech), plated onto SD medium lacking tryptophan and leucine,
and after incubation at 30°C for 4 days, screened for LacZ activity using
a colony lift filter assay. Putative interacting clones were isolated
by manipulation in leuB E. Coli, and further tested against pGBT9 and
control plasmids. A positive B-gal reaction between pGBT9/bcl-2 and
clone F1 was obtained using Sequenase and by automated dideoxy
sequencing. Additional clones containing fragments of F1 or human beclin
fused to the GAL4-activation domain were constructed using PCR primers
which incorporated Eco RI and Sal I restriction sites into the forward
and reverse primers, respectively.

Additional yeast two hybrid studies were performed to confirm

that human beclin, like mouse beclin, encodes a protein that

interacts with human Bcl-2, and to further define the

Bcl-2-interacting region of human Beclin (see Table 1).

Additional clones containing fragments of F1 or human beclin

fused to the GAL4-activiation domain were constructed using

PCR primers which incorporated Eco Ri and Sal I restriction sites into the forward and reverse primers, respectively.

Sequencing Of Human Beclin. Primers immediately upstream and
5 downstream of the predicted open reading frame were used to amplify the coding sequence of human *beclin* from a normalized human infant brain cDNA library (Soares, 1994). The resulting PCR products from several independent reactions were cloned into pCR^mII and sequenced in both directions using
10 Sequenase (US Biochemicals) as well as automated sequencing. The resulting nucleotide sequence (Figure 1B) and deduced amino acid sequence (Figure 1A) were used to scan various data banks (Genbank, EMBL, SwissProt, PIR) for homologous sequences using the BLAST algorithms (Altschul, 1990). The
15 amino acid sequence was also analyzed by the PROSITE program to identify functional motifs and by the COILS program to identify coiled coil regions (Lupas, 1991).

Yeast Two Hybrid Analyses of Beclin-Bcl-2 Family Member
20 **Interactions.** To investigate whether Beclin interacts with other Bcl-2 family members that positively or negatively regulate apoptosis, *bax*, *bcl-x$_s$* and *bcl-x$_L$* cDNAs were fused into the GAL4 binding domain vector and tested for interactions with Beclin in the yeast two hybrid system. (See
25 Table 1).

The sequences encoding amino acids 1 - 218 of human *bcl-2*, 1-212 of *bcl-x$_L$*, 1-149 of *bcl-x$_s$*, and 1-171 of *bax* were cloned into pGBT9 in frame with the GAL4-binding domain. To
30 avoid problems with targeting of proteins to the nucleus, the sequences encoding C'terminal transmembrane domains were omitted. To construct pGBT9/*bcl-2*, human *bcl-2* was amplified by PCR from the plasmid pZIP/*bcl-2*, subcloned into pCR^mII, and the correct sequence of *bcl-2* was confirmed prior to
35 cloning an Eco RI-Sal I fragment into pGBT9. To construct

pGBT9/bcl-x$_L$, pGBT9/bcl-x$_S$, and pGBT6/Bax, the Eco RI – Xho
I fragments were excised from pGEG202 plasmids previously
described (Sato, et al., 1994) and cloned into the Eco RI –
Sal I sites of pGBT9. Control pGBT9 plasmids containing
5 lamin (pLAM5') and p53 (pVA3) inserts were obtained from
Clontech.

The Bcl-x$_L$ GAL4 DB construct activated transcription by
itself, and therefore could not be tested for interactions
10 with Beclin. The same region of Beclin (aa 88-150) that
interacted with Bcl-2, also interacted with Bcl-x$_L$ (Boise,
1993), a related Bcl-2 family member that inhibits apoptosis.
In contrast, Beclin did not react with Bax (Oltvai, 1993),
a family member that promotes apoptosis. The selective
15 interaction of Beclin with Bcl-2 family members that have
death repressor activity suggests a possible functional role
of Beclin in anti-apoptotic pathways.

Full-length human Beclin does not interact with Bcl-2 in the
20 yeast two-hybrid system. This most likely reflects lack of
translocation to the nucleus in yeast secondary to
association with yeast intracellular membranes since
full-length human Beclin expressed in mammalian cells is
associated with the insoluble membrane fraction after cell
25 lysis.

To   evaluate   whether   Bcl-2-Beclin   and   Bcl-x$_L$-Beclin
interactions are related to the ability of Bcl-2 and Bcl-x$_L$
to inhibit apoptosis, pGBT9 vectors were constructed
30 containing bcl-2 and bcl-x$_L$ constructs with mutations in the
conserved BH1 domain that are known to block death repressor
activity.   A G->A mutation at amino acid position 145 of
Bcl-2 completely abrogates Bcl-2 death-repressor activity in
interleukin-3   deprivation,   γ-irradiation   and
35 glucocorticoid-induced apoptosis (Yin, 1994), and also blocks
Bcl-2 binding to beclin in the yeast two hybrid system (Table

2). Similarly, substitutions of amino acids 136-138 of Bcl-x$_L$ (VNW-->AIL) completely abolishes death repressor activity in Sindbis virus-induced apoptosis (Cheng, 1996), and also blocks Bcl-x$_L$ binding to Beclin. Thus, mutations that block anti-death activity of bcl-2 and bcl-x$_L$ also block binding to beclin.

Table 2.   **Effect of BH1 domain mutations on the ability of Bcl-2 and Bcl-x$_L$ to bind to beclin in the yeast two-hybrid assay**

		Inhibition of Apoptosis	Beclin Binding
WT BCL-2	ELFRDGVNWGRIVAFFEFGG	+	+
WT BCL-X$_L$	ELFRDGVNWGRIVAFFSFGG	+	+
MT BCL-2	---------A----------	-	-
MT BCL-X$_L$	------AIL-----------	-	-

Oligonucleotide-directed mutagenesis of *bcl-2* and *bcl-x$_L$* was accomplished by a two-step polymerase chain reaction. Mutants were cloned into pCR^{TMII} (In Vitrogen) and mutations were confirmed by dideoxy sequencing prior to cloning into pGBT9. PGBT9/*bcl-2* and pGBT0/*bcl-x$_L$* mutants were cotransformed with fragments of human *beclin* (1-450, 262-450, 1-708) fused to the GAL4-activation domain. Transformants were screened by B-galactosidase assays to determine whether mutations affected Beclin binding.

Analysis Of Beclin Expression In Mammalian Cells. Human *beclin* is predicted to encode a novel 450 amino acid protein, containing a coiled coil region with 25-28% homology with myosin-like proteins (Figure 1A). Western blot analysis of
5 lysates prepared from BHK cells infected with a Sindbis virus vector that expresses flag epitope-tagged Beclin confirms that human *beclin* encodes a 60 kd protein (Figure 2A).

To construct the plasmid SIN/flag-*beclin*, human *beclin* was
10 amplified by PCR from a human brain cDNA library, using primers that incorporated upstream and downstream Bst EII sites and an upstream sequence encoding the flag epitope. The Bst EII flag-*beclin* fragment was ligated into the Bst EII restriction site of the previously described double
15 subgenomic SIN vector, ds633. Recombinant virus stocks were generated from SIN/flag-*beclin* plasmid as described. BHK cells were infected with SIN/flag-*beclin* or control constructs at a multiplicity of infection (MOI) of 1 plaque-forming unit per cell and harvested 15 hours after
20 infection.

PROSITE analysis of human beclin identified several potential phosphorylation and myristoylation sites, but no other functional sequence motifs. RNA blot analysis revealed that
25 expression of *beclin* mRNA is widespread in both mouse and human adult tissues. A *beclin*-specific probe hybridized to a 2.3 kb transcript present at highest levels in human skeletal muscle, but at detectable levels in all tissues examined (Figure 2B). In some tissues, additional 1.7 and
30 1.4 kb transcripts were observed, suggesting the presence of alternately spliced transcripts.

To examine the subcellular localization of Beclin in mammalian cells and to determine whether Beclin colocalizes
35 with Bcl-2, baby hamster kidney cells were transmitted with

the plasmids, pSG5/*bcl-2* and pSG5/*beclin*, that express Bcl-2
and   a   flag-epitope   tagged   Beclin,   respectively.
Immunofluorescence   staining   with   an   anti-flag   epitope
antibody and an anti-Bcl-2 antibody revealed that both

5 proteins   were   expressed   in   the   perinuclear
membrane/endoplasmic reticulum region (Figure 2C). Confocal
laser microscopic analysis of BHK cells co-transfected with
flag-epitope-tagged Beclin and Bcl-2 revealed that Beclin
(Figure 2D), like BCL-2 (Figure 2C), displays a punctate

10 pattern of immunoreactivity that is characteristic of
association with intracellular organelles. Thus, Bcl-2 and
Beclin colocalize in transfected mammalian cells.

Beclin's Ability To Interact With Bcl-2. When expressed in
15 transient transfection assays, flag-tagged full-length human
Beclin displays punctate immunoreactivity pattern suggestive
of   association   with   intracellular   organelles   and   is
associated with the insoluble fraction after cell lysis. In
contrast, a flag-tagged truncated Beclin (a.a. 1-236)

20 (corresponding to the region isolated in the yeast two hybrid
screen) displays a diffuse cytoplasmic staining pattern and
is soluble after cell lysis (Liang and Levine, unpublished
data). These differences between full-length and truncated
Beclin are thought to account for differences in ability to

25 translocate to yeast nuclei and interact with Bcl-2 in the
yeast two hybrid assay.

The ability of human Beclin to bind to Bcl-2 in the yeast
two-hybrid system maps to amino acids 88-150 (nucleotides
30 262-450). Interestingly, the coding sequence for this region
of Beclin is deleted in some human infant brain cDNA clones
in the Merck EST database, suggesting that Beclin exists in
at least two forms-including one form that contains a Bcl-2
binding domain and one form that lacks a Bcl-2 binding

35 domain.

To examine whether a full-length human Beclin interacts with
Bcl-2 in mammalian cells, a fluorescence resonance energy
transfer (FRET) studies of COS cells co-transfected with Bcl-
2 and flag epitope-tagged Beclin was performed. Beclin is

5   a coiled coiled protein that may be associated with the
cytoskeleton and it partitions with the insoluble fraction
following cell lysis. For this technical reason, biochemical
analyses of *in vivo* interactions between Bcl-2 and Beclin are
difficult to perform. FRET is a flurorescence technique

10 which can be used as a spectroscopic ruler to study and
quantify the interactions of cellular components with each
other (Stryer, L., 1978; Wu, P. and Brand, L., 1994; Wang,
S.F., et al., 1993; Selvin, P.R., 1995; Gadella, T.W., 1994).
In FRET, a fluorophor (donor) in an excited state may

15 transfer its excitation energy to a neighboring chromophor
(acceptor) nonradiatively through dipole-dipole interactions.
The efficiency of this process varies most importantly as the
inverse of the sixth power of the distance separating the
donor and acceptor chromophores, and in practice, requires

20 the distance between the donor and the acceptor flurophores
to be close (usually not exceeding 50 Angstroms). The
d pendence of the energy transfer efficiency on the donor-
acceptor separation provides the basis for the utility of
this phenomenon in the study of cell component interactions.

25 FRET has been used by a number of investigators to examine
interactions of cellular constitutents (reviewed in Stryer,
L., 1978; Wu, P. and Brand, L., 1994; Wang, S.F., et al.,
1993; Selvin, P.R., 1995; Gadella, T.W., 1994) such as
endosomal fusion events, ligand-dependent growth factor

30 receptor aggregations, interactions of viral and cellular
proteins with regulators of apoptosis (Liang, X.H., et al.,
1994; Mahajan, N., et al., 1996; Herman, B., et al., 1997),
and interactions of cellular cytoskeletal components (Root,
D., 1997).

35

FRET can be detected by exciting the labeled specimen with
light of wavelengths corresponding to the absorption spectrum
of the donor and then detecting light emitted at the
wavelengths corresponding to the emission spectrum of the
acceptor. FRET manifests itself by both quenching of donor
fluorescence in the presence of acceptor and in sensitized
emission of acceptor fluorescence. FRET microscopy was
performed as described herein. The donor (FITC) filter set
consisted of excitation (ex)=450-490 nm; dichroic mirror
(dm)=510nm; emission (em)=515-555 nM. The acceptor
(Rhodamine) filter set consisted of [ex=546 nm; dm=580 nm;
long pass]. Images obtained with these two filter sets were
used to directly quantify the intensities of each
fluorophore. The FRET filter set consisted of [ex=450-490
nm; dm=580 nm; em=580 long pass]. The signal recorded from
this filter set is the FRET signal and is from energy that
has transferred from FITC to Rhodamine molecules. A
background image containing no cells was taken with each
filter set and subtracted from images with cells. A mapping
program written in house was used to map fluorescent cells
and to quantify the intensity within each cell. Quantitative
analysis of these mapped images required solving three
equations, one for each filter set, which accounted for the
excitation and detection of both labels in all three filter
sets as well as the concentrations of the donor and acceptor
molecules and the probability of energy transfer. The
measured quantities are expressed as follows in which the
first letter (upper case) indicates the filter set (A =
acceptor; F=FRET; D=donor) and the second letter (lower case)
indicates the labels preset (a=acceptor alone; f=acceptor and
donor; d=donor alone). A solution of the equations is
$E=1/[aconc(RK=1)]$ where $aconc=(AdFf-FdAf)/[(AdFa/Aa)-Fd]$;
$R=(DaFf/Fa-Df/[aconc ((Fa/Aa)-FdDa/DdAa)-Ff+FdDf/Dd]$; and K
is proportional to the product of the ratio of the quantum
yield of the two labels and the ratio of the absolute
detection efficiencies of the two labels.

Flag epiope-taged Beclin was labled with donor fluorophor
(FITC) and Bcl-2 was labeled with acceptor fluorophor
(Rhodamine) (Figures 3A-I). As a control, the endoplasmic
reticulum Ca^{2+} -ATPases (SERCA) with donor (FITC) and Bcl-2
with acceptor (Rhodamine) were used. Quantitative analysis
of microscopic images (following corrections for cross-talk
between filter sets and donor and acceptor concentrations)
showed significantly more energy transfer in experimental
cells with labeled Beclin and Bcl-2 than in control cells
with labeled SERCA and Bcl-2. (The quantitative measure of
FRET used is a value, E, proportional to the probability of
energy transfer between any donor molecule and any acceptor
molecule). For the experimental cells, $E_{beclin-Bcl-2}$ = 0.00325
±0.00153 (N=410) and for the control cells, $E_{SERCA-Bcl-2}$ =
0.00065 ± 0.00043 (N=775) (p=<0.0001; t test). These data
demonstrate that Beclin and Bcl-2 exhibited FRET, providing
evidence of an interaction between these two proteins in
mammalian cells.

Role Of Beclin In Virus-Induced Apoptosis. Overexpression
of many Bcl-2 family members (Boise, 1993; Oltvai, 1993) or
Bcl-2 interacting proteins (Farrow, Takayama) results in
either the acceleration or inhibition of apoptosis. The
5 Sindbis virus vector system was employed, which has been
previously used to study the anti-apoptotic function of
several Bcl-2 family members (Cheng, 1996), to evaluate the
effects of beclin overexpression on virus-induced apoptosis.
This system uses Sindbis virus both as an inducer of
10 apoptosis and as a vector, and provides a means of rapidly
testing the ability of candidate death-regulatory genes to
suppress or accelerate virus-induced apoptosis (Cheng, H.,
et al., 1996); Levine, B., et al., 1996). Chimeric
recombinant Sindbis viruses were constructed that contain
15 human beclin or bcl-2 inserts in the sense and antisense
orientations. Bcl-2 expressed from the recombinant Sindbis
virus delayed death in BHK cells, as indicated by 50% cell
viability compared to 0% with control viruses at 30 hours
post-infection. However, neither Beclin nor antisense beclin
20 expressed from recombinant viruses delayed or accelerated
virus-induced death (Figure 4A). While Bcl-2 overexpression
results in a significant delay in SIN-induced cell death of
BHK cells, neither antisense beclin RNA nor beclin
overexpression delays or accelerates virus-induced death.
25 Therefore, rather than acting as an independent regulator of
apoptosis, Beclin may be a functional component of a pathway
that is mechanistically involved in the death repressor
activity of Bcl-2.

30 **Role Of Beclin In Cellular Proliferation.** To test this
hypothesis, Beclin was tested to see if it plays a role in
the ability of Bcl-2 to inhibit virus-induced apoptosis in
mammalian cells.   A *bcl-2-* transfected rat prostate
adenocarcinoma cell line (AT3/bcl-2 cells) that is resistant
35 to Sindbis virus-induced apoptosis (Levine, 1996) was

infected with chimeric Sindbis viruses containing *beclin* in
either the sense or antisense orientation.

To construct the plasmid SIN/flag-*beclin*, human *beclin* was
amplified by PCR from a human brain cDNA library, using
primers that incorporated upstream and downstream Bst EII
sites and an upstream sequence encoding the flag epitope.
The Bst EII flag-*beclin* fragment was ligated into the Bst EII
restriction   site   of   the   previously   described   double
subgenomic SIN vector, ds633, and recombinant virus stocks
were generated from SIN/flag-*beclin* plasmid as described.
The   recombinant   chimeric   viruses   SIN/antisense   *bcl-2*
SIN/antisense   *beclin*   were   constructed   using   identical
methods.   The construction of SIN/CAT has been described
previously.

At ·72 hours after infection, 77% of cells infected with
SIN/*beclin* and 66% of cells infected with a control chimeric
virus, SIN/CAT were still alive (Figure 4B).   In contrast,
only 35% of cells infected with SIN/antisense *beclin* were
still alive.   The magnitude of this increase in cell death
is   similar   to   that   seen   after   infection   with   a   virus
containing *bcl-2* antisense RNA.   The ability of antisense
beclin, like antisense *bcl-2*, to partially inhibit bcl-2
protection   against   Sindbis   virus-induced   apoptosis
demonstrates   a   functional   role   for   Beclin   in   the   death
repressor activity of Bcl-2.

In   the   course   of   the   above   experiments,   an   apparent
inhibition of cellular proliferation in both BHK cells and
AT3/*bcl-2* cells infected with SIN/*beclin* was observed.   The
number   of   AT3/*bcl-2*   cells   24   hours   after   infection   with
SIN/*beclin* was reduced by more than 50% as compared to the
number of AT3/*bcl-2* cells that were mock-infected or infected
with SIN/CAT (Figure 5A), whereas no significant differences

were observed in AT3/*bcl-2* cell viability among the three
groups (Figure 5B). A similar antiproliferative effect of
beclin was observed in control AT3/neo cells, although these
cells die quickly after infection with SIN/beclin and it is
5 difficult to accurately quantitate cell proliferation in the
face of rapid cell death.

These findings were confirmed in AT3 cells using a different
experimental system, mouse fibroblast NIH 3T3 cell lines that
10 conditionally express beclin or antisense beclin, using
tetracycline-repressible expression vector.

The vectors were made by amplifying a full length human
beclin from a human brain cDNA library (Soares, M.B., 1994)
15 using PCR primers which incorporated Nhe I resriction sites,
and cloned into the Nhe I cloning sites of the conditional
expression vector, BC252SV40 (provided by Axel Pollack, GSF-
Institut fur Klinische Molekularbiologie). BC252SV40
consists of pHEBO, an EBV-derived promoter (TP1) in which the
20 EBNA2 binding site was replaced by the tet07 element and an
expression cassette for the tTA transactivator under the
control of a CMV promoter, and a hygromycin selection marker.
tTA binds to tet07 in the absence of tetracycline, but not
in its presence, and induced the transcription of genes under
25 control of tet07. The plasmids BC252SV40, BC252SV40/beclin,
and BC252SV40/antisense beclin were transfected into NIH 3T3
cells using PFX6 (inVitrogen) according to the manufacturer's
instructions, and stable transfectants were selected for with
300 µg/ml hygromycin and maintained in 1 µg/ml tetracycline.
30 Antisense beclin-transfected clones were screed for inducible
antisense flag-beclin mRNA expression by performing RT-PCR
in the presence and absence of tetracycline, using a forward
primer that hybridizes with the nucleotide sequence encoding
the flag epitope tag and a reverse primer corresponding to
35 nucleotides 746-765 of human beclin. Beclin-transfected
clones were screened for inducible flag-beclin mRNA

expression by RT-PCR and flag-Beclin protein expression by
performing immunoblot analysis with an anti-flag M2 antibody
(20 μg/ml) using previously described methods (Liang, X.H.,
1995).

5

A tetracycline-responsive NIH 3T3 clone stably transfected
with antisense beclin and a control NIH 3T3 clone transfected
with an empty vector were infected with recombinant chimeric
Sindbis viruses that express either wild-type Bcl-2 (SIN/bcl-
10   2) or Bcl-2 containing a stop codon near the 5' terminus
(SIN/bcl-2 stop) (Figure 4C).   In the presence of
tetracycline   (when   antisense   beclin   expression   is
suppressed), protection against virus-induced death was
observed in both SIN/bcl-2 infected NIH/antisense beclin
15   cells as well as in control cells. However, in the absense
of tetracycline (when antisense beclin is expressed), no
protection against Sindbis virus-induced death was conferred
by bcl-2 in NIH 3T3/antisense beclin cells. Thus, similar
to the observation in AT3 cells, antisense beclin expression
20   also blocks the death repressor activity of bcl-2 in NIH 3T3
cells.

Cell proliferation of tetracycline-responsive NIH 3T3/beclin
and NIH 3T3 antisense beclin clones and NIH 3T3 control
25   transfectants in the presence or absence of tetracycline was
measured (Figure 5C). Tetracycline had no effect on the
proliferation of control NIH 3T3 cells transfected with empty
vector.   In the presence of tetracycline, NIH 3T3/beclin
cells and NIH 3T3/antisense beclin cells proliferated at the
30   same rate as control transfectants.   However, when
tetracycline was removed from the culture medium, the rate
of cell growth was significantly delayed in beclin-expressing
cells and conversely, was accelerated in antisense beclin-
expressing cells. These data demonstrate a role for beclin
35   in the regulation of NIH 3T3 cell growth.

In view of genetic data suggesting that chromosome 17q21
contains a second tumor suppressor gene (in addition to
BRCA1) important in sporadic breast and ovarian cancer
(reviewed in Tangir, J., et al., 1996; Vogelstein, B., et
5   al., 1994), the effect of beclin gene transfer on human MCF7
breast carcinoma cells was evaluated. Wild-type MCF7 cells
(which are derived from a tumor containing 17q21 LOH) (Holt,
J.T., et al., 1996) were transfected with the BC252SV40
expression vector containing wild-type Beclin and hygromycin-
10   resistant clones were selected.   As expected after
transfection with a gene that may negatively regulate cell
growth, in several independent transfections, very few
hygromycin-resistant colonies were obtained that grew beyond
10-20 cells and which could be tested for gene expression.
15   Among five hygromycin-resistant colonies that could be
expanded, four had flag-Beclin message RNA detectable by RT-
PCR but only one of these clones had detectable flag-Beclin
expression by Western blot analysis (clone 17) (Figure 6A).
The tetracycline-regulatable expression vector, BC2525V40
20   previously described was used for MCF7 transfections with
beclin.   However, no tetracycline-responsive hygromycin
resistant MCF7 Beclin clones were obtained; result are
presented using MCF7 Beclin clone 17 which constitutively
expresses flag-Beclin. This clone was chosen for further
25   analysis.

Morphologic changes suggestive of a less transformed
phenotype were observed in MCF7 cells that express Beclin.
Whereas control cells were small, refractile, and often
30   rounded (Figure 6B), MCF7 Beclin cells were significantly
larger, more flat in appearance and more firmly attached to
the tissue culture plate (Figure 6C). Control cells grew to
a higher density (Figure 6B), while cells that expressed
Beclin were contact-inhibited and grew as a monolayer in
35   culture (Figure 6C).

                                     -67-

Similar morphologic changes were observed in many MCF7
colonies obtained after transfection with BC2525V40/*beclin*
which could never be expanded for analysis of gene expression
and further investigations, but not in MCF7 colonies obtained
5   after transfection with the empty BC2525V40 vector.

To further evaluate anchorage-independent growth, the ability
of MCF7 Beclin 17 and MCF7 control 5 clones to form colonies
in soft agar was examined (see Table 3). Whereas MCF7 Beclin
10 17 cells were unable to form colonies in soft agar, the
number of colonies formed by MCF7 control 5 cells averaged
over 1100 per 5 x 10^4 cells plated.

Table 3.   Effect of beclin transfection on MCF7 colony
            formation in soft agar and tumorigenicity in
            nude mice.

Clone	# colonies ± s.e.	# mice w/ tumors/# mice injected	mean tumor volume (mm³) ± s.e.	mean tumor weight (mg) ± s.e.
MCF7 Beclin 17	0 ± 0	2/14*	92 ± 17 **	80 ± 0 **
MCF7 control 5	1047 ± 189	8/9	375 ± 135	360 ± 200
MCF7 control 7	ND	6/8	641 ± 178	490 ± 200
MCF7 control 9	ND	4/6	530 ± 100	490 ± 300

* p = 0.002 vs. MCF7 control clones; Chi-square analysis
**p = NS

Soft agar assays were performed as described (Jiang, W., et al., 1993)
and results are presented as the means ± s.e. number of colonies per 5
x 10⁴ plated cells/well for six wells. For tumorigenicity assays, five
week-old female CD1nu/nu mice (Charles River Laboratories) were implanted
with 1.7 mg estrogen/60 day release pellets (innovative Research of
America) and injected subcutaneously with 5 X 10⁶ MCF7 tumor cells. Mice
were monitored biweekly for thedevelopment of tumors and tumor size was
measured in two dimensions (length [a] and width [b]). Tumor volume was
calculated according to V=ab²/2. Mice were necropsied after eight weeks,
and mean tumor volumes and weights were determined fro the subgroup of
mice with tumors present upon necropsy.

The ability of Beclin to suppress clonigenicity in soft agar
assays suggested that it may also suppress tumorigenicity *in
vivo*. Therefore, the ability of MCF7 Beclin 17 and three
control clones to form tumors in five week old female nude
mice implanted with slow-release pellets was compared. After
an eight-week observation period, 66-77% of mice injected
with control clones developed autopsy-confirmed tumors, as
compared with only 14% of mice injected with Beclin-
expressing cells, demonstrating that Beclin expression
significantly decreases the incidence of tumor formation.
The tumors that did develop in mice injected with MCF7 Beclin
17 cells tended to have reduced tumor volume and weight
compared to those in mice injected with control MCF7 clones,
but these differences were not statistically significant.

No difference were observed with respect to the rate of tumor
growth or tumor histology.

In summary, the yeast two hybrid system was used to isolate
a cDNA that encodes a predicted coiled coil protein, Beclin,
that interacts with members of the Bcl-2 family that
negatively regulate apoptosis. A functional role for Beclin
in anti-apoptotic pathways is suggested both by Bcl-2 and
Bcl-x_L mutational studies showing a correlation between
disruption of anti-apoptotic function and binding to Beclin,
and by studies in which beclin antisense RNA partially blocks
Bcl-2-mediated protection against virus-induced apoptosis.
While the function of beclin, when expressed at normal levels
in mammalian cells, is still unknown, its overexpression can
inhibit cellular proliferation. These observations are
consistent with the hypothesis that Bcl-2 may inhibit
apoptosis by interacting with a gene product that exerts
effects on cellular proliferative machinery. Furthermore,
these findings, coupled with previous studies that have
mapped beclin transcripts to a breast and ovarian cancer
susceptibility locus on chromosome 17q21 (Rommens, 1995;
Friedman, 1994; Friedman, 1995), warrant additional
investigation to determine whether beclin, and its
interactions with Bcl-2, play a role in human cancer.

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SECOND SERIES OF EXPERIMENTS

Sequencing of 5' and 3' nontranslated regions of human beclin. The 5' and 3' untranslated sequences of human *beclin*

5   may be obtained by performing RACE using the Clontech
MARATHON KIT and MARATHON-READY mammary cDNA.   The 5'
untranslated sequence consists of 120 bp and is shown in
Figure 8A.   The 3' untranslated sequence consists of 654 bp
and is shown in Figure 8B.

10

**Identification of aberrant beclin transcripts in sporadic
ovarian cancer.**   To determine whether *beclin* fulfills the
criteria of a tumor suppressor gene important for ovarian
cancer (e.g. functional inactivation of both *beclin* alleles),

15   reverse transcriptase-PCR of RNA obtained from four sporadic
epithelial ovarian cancers with monoallelic deletions of the
beclin-spanning region of chromosome 17q21 and from four
normal ovaries was performed.   As one allele of *beclin* is
already deleted in these ovarian tumors, the demonstration

20   of an inactivating mutation on the remaining present allele
would fulfill the Knudson two hit hypothesis criteria
(Knudson et al., 1985) and constitute proof that *beclin* is
a tumor suppressor gene.

25   In all four of the ovarian tumors with deletions of one
beclin allele, sequence analysis of PCR-amplified *beclin* cDNA
products revealed heterozygosity in the region of *beclin*
between nucleotides 1042 and 1185.   To further evaluate this,
the PCR products were cloned into the TA cloning vector, and

30   several individual subclones for each sample (N>10) were
sequenced. Analysis has been completed of three of the four
ovarian tumor samples and four normal ovaries. All three of
the ovarian cases had aberrant splice products present,
whereas none of these splice products were found in the four

35   normal ovaries.   In addition, these splice products have not

been detected (using a similar approach) in RNA extracted
from normal brain, normal colon, normal breast, or five
different tissue culture cell lines. The splicing patterns
detected in the three ovarian cancer cases are depicted
5   schematically in Figure 9A. In all instances, the splicing
variation occurred beginning at nucleotide 1042 and resulted
in a premature termination codon. Three different patterns
were observed simultaneously in one case (deletion of
nucleotides 1042-1185 with resulting frameshift/stop codon;
10 intronic insertion after 1042, with deletion of 1042-1185,
with resulting frameshift/stop codon; and intronic insertion
between nucleotides 1041 and 1042 with resulting
frameshift/stop codon). The other two tumors had only
deletions of 1042-1185. This type of aberrant splice pattern
15 is highly suggestive of a splice site mutation at either the
acceptor splice site upstream of the exon beginning at 1042
or at the donor splice site downstream of the exon ending at
nucleotide 1185. Studies are in progress (see below) to
sequence these splice junction regions in genomic DNA from
20 the same tumor samples to identify the precise genomic
mutations responsible for this aberrant *beclin* splicing.
Such genomic DNA mutations will likely be identified in this
region of *beclin*; such a finding would further confirm that
beclin is an important tumor suppressor gene for sporadic
25 ovarian cancer.

**Altered subcellular distribution of aberrant beclin cDNA
transcripts found in ovarian cancer.** To begin to compare the
phenotype of the alternative *beclin* splice products found in
30 ovarian tumors (described in the previous section) with
full-length wild-type *beclin*, flag epitope-tagged *beclin*
deleted of nucleotides 1042-1185 (flag-beclinΔ1042-1185) and
flag-wild-type *beclin* were cloned into the mammalian
expression vector, pSG5. After transfection of monkey COS-7
35 cells and human breast carcinoma MCF7 cells, the pattern of

immunostaining   of   flag-BeclinΔ1042-1185   differed
significantly from flag-wild-type Beclin. Wild-type Beclin
is found exclusively in the cytoplasm, predominantly in the
perinuclear   region,   except   for   G2/M-specific   nuclear
5   localization in MCF7 cells. In contrast, BeclinΔ1042-1185
is found diffusely in both the cytoplasm and the nucleus in
asynchronous COS and MCF7 cells (see Figure 10). The ability
of this aberrant isoform to enter the nucleus in a cell
cycle-independent   fashion   (versus   cell   cycle-dependent
10   fashion of wild-type Beclin) may have important implications
for potential mechanisms by which it might have different
biologic effects than full-length Beclin. Studies are in
progress to directly compare the tumor suppressor function
of BeclinΔ1042-1185 with wild-type Beclin.

15

Loss of Beclin expression in invasive breast carcinoma. To
examine whether Beclin may be functionally inactive in human
sporadic breast cancers, a comparison was done of the levels
of Beclin protein expression in matched normal and malignant
20   breast tissue from the same cases, using a polyclonal
anti-Beclin peptide (aa 1-15) antibody, 843 (N=20). As shown
in the Western blot in Figure 11, 843 reacts specifically
with a 61 kd protein in human breast tissue that migrates
identically on SDS-Page gels to human Beclin expressed in
25   transfected cells.   By Western blot analysis, 15 of 17
samples with detectable Beclin in normal tissue had absent
(N=7) or significantly reduced Beclin (N=8) in the tumor
tissue.   (Some normal tissues were negative presumably due
to the absence or limited number of epithelial cells in the
30   tissue sample; the absence of protein in these samples was
confirmed by Western blot analysis of actin expression.).
Since equivalent volumes of normal and tumor tissue (rather
than equivalent amounts of extracted protein) were analyzed,
and normal tissue contains primarily fat cells whereas tumor
35   tissue contains primarily epithelial cells, Western blot

analysis using an anti-actin antibody confirmed that tumor
samples   invariably   contained   significantly   higher
concentrations of protein.   Thus, the decreased level of
Beclin protein expression in tumors cannot be explained by
5   a decrease in the amount of total protein loaded.

To confirm that the decreased Beclin protein expression in
tumors observed on Western blot analysis reflected loss of
expression in epithelial cells, immunocytochemical staining
10 was performed to detect Beclin protein using antibody 843 in
paraffin-embedded sections from 10 of the matched normal and
malignant breast samples analyzed by Western blot.   In all
10 cases, there was strong Beclin immunoreactivity in all
normal breast epithelial cells.   However, in 5 of the 10
15 cases, Beclin immunoreactivity was completely absent in the
malignant epithelial cells.   (An example of Beclin staining
in normal epithelia but not malignant epithelia from one
representative case of breast cancer (patient 516) is shown
in Figure 12.)   Thus, these findings suggest that Beclin
20 protein expression is lost in about half of invasive breast
cancer cases, and are consistent with the genetic evidence
that *beclin* is a tumor suppressor gene important for breast
cancer.

B.   Studies in progress

Sequencing of *beclin* splice site junctions in ovarian, breast, and prostate cancer.   The detection of aberrant *beclin* splice products in ovarian cancer (see Figure 9),

5   suggests that one mechanism of *beclin* inactivation in ovarian, breast, and/or prostate tumors is through mutations in the splice site regions at the 5' or 3' intron-exon boundaries of the *beclin* exon spanning from nucleotide 1042-1185.   This possibility may be evaluated by amplifying

10 by PCR *beclin* genomic DNA fragments spanning the exon-exon boundaries at 1041/1042 and at 1185/1186 from ovarian tumors containing 17q21 LOH and from peripheral blood from the same patients and performing DNA sequencing of the PCR products. Breast and prostate tumor samples from 17q21 LOH may be

15 screened, and once 17q21 LOH samples are identified, such samples may be subjected to the same analyses to detect similar splice site mutations in breast and prostate samples. It is anticipated that these analyses will identify somatic mutations in ovarian, breast, and prostate tumors in *beclin*

20 splice site regions.   Furthermore, it is predicted that these somatic mutations will lead to aberrant *beclin* cDNA transcripts which have loss-of-tumor suppressor function. The presence of aberrant *beclin* cDNA transcripts in samples containing *beclin* splice site mutations may be confirmed by

25 sequencing RT-PCR-amplified *beclin* RNA, and the loss-of-tumor suppressor function of such transcripts may be confirmed by studying their ability to suppress the ability of human breast carcinoma MCF7 cells to form tumors in nude mice.

Sequencing of beclin 5' and 3' untranslated regions (UTR's),
DNA in ovarian, breast, and prostate cancer samples with
monoallelic deletions of beclin. The observation that Beclin
protein expression is absent in about 50% of cases of breast
5 cancer supports the hypothesis that mutations may occur in
cancer in regions of the beclin gene that regulate levels of
expression. Such regions may include the 5' and 3' UTRs and
the promoter and enhancer regions. To identify potential
mutations in the 5' and 3' UTRs of beclin, genomic DNA
10 extracted from ovarian, breast, and prostate cancers
containing 17q21 LOH may be amplified by PCR using primers
that span either the 5' UTR or 3' UTR and the PCR products
may be sequenced. Genomic DNA from peripheral blood from the
same cases may be analyzed in a similar fashion to detect
15 whether any somatic mutations are present in the tumor DNA.
It is anticipated that these studies will identify mutations
in the 5' or 3' NTR regions of human beclin in breast,
ovarian, and/or prostate cancer samples which lead to
decreased levels of Beclin protein expression in the tumors.
20

Sequencing of beclin promoter and enhancer regions in
ovarian, breast, and prostate cancer samples with monoallelic
deletions of beclin. Sequences of complete genomic Beclin
may be determined. From the sequence of the full-length
25 beclin gene, available software may be used for
identification of potentially important regulatory sequences,
including promoters and enhancers. (e.g. Reese's neural
network algorithm for identifying promoters, MOTIFS type
software for identifying elements often occurring within
30 enhancers and promoters, and other public and commercial
programs maintained in packages compiled by the Columbia
Genome Center Informatics Section). One may perform mutation
analysis of all beclin regulatory sequences in genomic DNA
from ovarian, breast, and prostate cancer samples with 17q21
35 LOH to detect mutations in the remaining allele. Genomic DNA

from the promoter and enhancer regions may be amplified by
PCR and PCR products may be sequenced. The sequences may be
compared to control DNA from normal cells for each patient
with cancer.   It is anticipated that these studies will
5   identify mutations in the promoter or enhancer regions of the
beclin gene that affect regulation of *beclin* gene expression
in breast, prostate, or ovarian cancer.

Analysis of methylation status of *beclin* DNA promoter in
10   **ovarian, breast, and prostate cancer samples with monoallelic**
deletions of *beclin*.   Aberrant methylation of normally
unmethylated CpG islands has been associated with
transcriptional inactivation of defined tumor suppressor
genes in human cancers, including p16, p15, E-cadherin, and
15   von Hippel-Lindau and is postulated to be the mechanism of
inactivation of · the tumor suppressor gene p73 in
neuroblastomas (Kaghad et al., 1997). Further experiments
will determine whether hypermethylation of the *beclin*
promoter occurs in the remaining allele in breast, ovarian,
20   and prostate cancer cases with 17q21 LOH. DNA extracted from
breast, ovarian and prostate cancer cases may be subjected
to the PCR assay for methylation status of CpG islands
described in the methods sections. It is anticipated that
these studies will find that the promoter region of *beclin*
25   is hypermethylated, thereby constituting an epigenetic
mechanism for silencing *beclin* expression in ovarian, breast,
and/or prostate cancers.

Mutation analysis of coding regions of beclin in ovarian, breast, and prostate cancer samples with monoallelilc deletions of beclin. Although the analyses completed to date on ovarian and breast cancer samples specifically suggest

5   mutations in splice site junctions or in regulatory regions of beclin, most tumor suppressor genes contain a variety of different types of mutations in different cancers. In addition, coding region mutations which lead to decreased protein stability could also explain the loss of Beclin

10   protein expression observed in human breast cancers. Therefore, it is predicted that beclin may also be mutated in the coding regions in some ovarian, breast, and prostate cancers. To detect coding region mutations in genomic DNA from ovarian, breast, and prostate cancers with 17q21 LOH,

15   each exon may be amplified by PCR separately using primers designed within the introns just beyond the splice sites and directed inwardly toward the exons. PCR products may be sequenced and compared to similarly amplified genomic DNA from peripheral blood. It is anticipated that these studies

20   will identify coding region mutations in beclin in ovarian, breast, and prostate cancer.

Functional analysis of beclin mutations. The functional inactivation of beclin on both alleles in certain types of

25   cancer would be definitive evidence that beclin is a tumor suppressor gene important in that type of cancer, and that such mutations may play a role in the development of that type of cancer. All of the mutational analyses performed above may be done using tumor samples that contain deletions

30   of one allele of beclin. Thus, demonstration of an inactivating mutation on the remaining allele would be sufficient to classify beclin as a tumor suppressor gene important for the type of cancer in which such a mutation is found. Inactivation mutations include mutations in

35   regulatory regions that lead to loss of Beclin protein

expression or mutations in the coding region which lead to premature stop codons. Such mutations are functionally important and constitute definitive evidence for a mechanism whereby mutations in *beclin* can lead to the development of
5 human cancer. The importance of mutations in the coding region that do not lead to premature stop codons may be determined by performing tumor suppressor functional assays with Beclin proteins containing such mutations. These assays may be performed as described in the methods section. One
10 may make breast carcinoma cell lines that conditionally express the mutated form(s) of Beclin, and compare the ability of these cell lines to form tumors in nude mice with the ability of cell lines expressing wild-type Beclin. These studies would help determine whether mutations observed in
15 human cancers inactivate the tumor suppressor function of *beclin*.

Mutational analyses of *beclin* in ovarian, breast, and prostate carcinoma cell lines. In addition to performing
20 mutation analyses of primary tumors containing 17q21 LOH, future experiments would also examine whether ovarian, breast, and prostate carcinoma cell lines contain mutations in *beclin* in either the regulatory regions, coding regions, or splice site junctions. An identical approach may be used
25 as that described above for tumor samples.

**G2/M specific nuclear localization of Beclin and Bcl-2 in
MCF7 cells.** Observations in MCF7 cells suggest that Beclin
has tumor suppressor activity in this cell type. To explore
potential mechanisms by which Beclin may regulate the growth
5 of MCF7 cells, it was investigated whether Beclin, like Bcl-
2, undergoes cell-cycle dependent nuclear localization. In
contrast to the usual pattern of association with cytoplasmic
membranes in non-epithelial cells, Bcl-2 expression has been
shown to increase during G2/M and to localize to mitotic
10 chromosomes in cultured SV40-immortalized normal mammary
epithelial cells and in epithelial cell lines derived from
breast, ovary, colon, cervical, and pancreatic carcinomas.

Immunocytochemical and DNA staining of asynchronous and G2/M-
15 arrested transiently transfected cells was performed to
determine whether Beclin and Bcl-2 co-localize to mitotic
nuclei in MCF7 cells.   Similar to findings reported
previously, very few MCF7 cells displayed any endogenous Bcl-
2 staining in interphase, and when observed, staining was
20 cytoplasmic.   In contrast, after taxol treatment (which
arrests cells in G2/M), the majority of MCF7 cells displayed
endogenous nuclear Bcl-2 staining. In MCF7 cells transfected
with Bcl-2 and flag-Beclin, the subcellular localization of
Bcl-2 and flag-Beclin immunoreactivity was identical in each
25 co-transfected cell and varied according to the stage of the
cell cycle.   In interphase cells, the pattern of
immunoreactivity for Bcl-2 and flag-Beclin was identical to
that described above in COS7 cells, with a punctate
cytoplasmic appearance. In contrast, untreated MCF7 cells
30 spontaneously traversing G2/M or taxol-treated MCF7 cells
arrested in G2/M displayed nuclear localization of Bcl-2 and
flag-Beclin. The pattern of Beclin staining was similar to
that reported previously for Bcl-2. Both Beclin and Bcl-2
display a diffuse nuclear pattern in G2 or early prophase,
35 co-localize with condensed chromosomes in late prophase and

metaphase, and display little to no staining in anaphase.
The intensity of the flag-Beclin-staining was greater in
mitotic than interphase cells and the number of transfected
MCF7 cells that displayed detectable flag-Beclin protein
5   expression was greater in taxol-treated vs. untreated cells,
suggesting an increase in Beclin stability in G2/M. Nuclear
localization or increased levels of flag-Beclin and Bcl-2
were not observed in co-transfected NIH3T3 cells treated with
taxol.   Thus, Beclin, like Bcl-2, undergoes G2/M-specific
10 nuclear import and enhanced protein stability in MCF7 cells,
but not in a non-epithelial cell line.

Identification of a Beclin Nuclear Export Signal. The cell-
cycle regulated nucleocytoplasmic transport of Beclin
15 suggested the examination of whether the leucine rich motif
of Beclin (containing a high degree of homology with the
nuclear export signals of PKI and HIV-1 Rev) encodes a
functional nuclear export signal. Based upon mutagenesis
studies with PKI nuclear export signal, one would predict
20 that alanine substitutions of the leucine residues at
positions 184 and 187 of Beclin would impair nuclear export.
This prediction was tested by performining immuno-
fluorescence studies of COS7, NIH3T3, and MCF7 cells
transfected with either wild-type flag epitope-tagged Beclin
25 or a flag epitope-tagged mutant Beclin containing alanine
substitutions at amino acids 184 and 187 (referred to as
NESM1 for nuclear export signal mutant 1). In contrast to
the exclusive cytoplasmic localization of wild-type Beclin
in interphase cells, Beclin NESM1 displayed both intense
30 punctate nuclear staining as well as cytoplasmic staining in
all three cell types. The ability of two leucine mutations
within a nuclear export signal motif to result in cell-cyle
independent nuclear localization of Beclin (even in cell
types which do not normally undergo cell-cycle dependent

nuclear localization) provides evidence that Beclin contains
a functional nuclear export signal.

METHODS

5

Rapid amplification of cDNA ends (RACE)   To isolate and
sequence the 5' and 3' untranslated regions of the human
beclin cDNA, the RACE method was performed using the MARATHON
cDNA amplification kit and MARATHON-READY human mammary cDNA
10   obtained from Clontech, according to the manufacturer's
instructions.

Preparation of tumor and control samples for DNA sequencing.
Total RNA from ovarian, breast, and prostate cancer and
15   control tissue and cell lines may be extracted using the
RNAzol method according to the manufacturer's instructions
(Teletest, Friendswood, Texas), reverse transcribed using
random hexamer primers, and PCR may be performed using
primers that amplify full-length human beclin DNA.   PCR
20   products may be sequenced as described in the next section.
Genomic DNA may be extracted using standard methods (Dracopli
et al. 1997) and sequenced as described in the next section.

25   **DNA sequencing.** Automated cycle sequencing using dye tagged
primers and terminators may be performed on PCR products
using methods established at the Columbia University Core DNA
Synthesis and Sequencing Facility. Primer walking, shotgun
cloning, and sequence assembly may be performed using methods
30   established in the Columbia Genome Center sequencing
laboratory. Mutational analysis may be conducted using the
methods established in the Columbia Genome Center laboratory
for heterozygote analysis on other projects.   Normal and
modified sequences may be compared using the SEQUENCE

NAVIGATOR software (Applied Biosystems, Inc., Foster City,
CAT).

Loss-of-heterozygosity (LOH) analysis.   LOH analysis of
chromosome 17q21 of ovarian tumor samples was performed by
5   the laboratory of Dr. Samuel Mok as described previously
(Tangier et al., 1996). To select breast and prostate cancer
cases for analysis, LOH studies may be performed on tumor DNA
from microdissected tumor samples and normal DNA from
peripheral tissue using the methods described by Tangir et
10 al.   A panel of eight highly polymorphic tandem repeat
markers on chromosome 17q21 (D17S1320, D17S1321, D17S1328,
D17S855, D17S1322, D17S1323, D17S1327 and D172136) may be
used. The order, map position, relative distance between the
markers, and relationship between the markers and *beclin* and
15 BRCA1 is already known. Cases which have LOH which encompass
beclin may be used for further analysis.

Methylation-specific PCR.   The PCR assay for methylation
status of CpG islands described in Herman et al. (Herman et
20 al. 1996) may be used to assess whether *beclin* genomic DNA
from primary tumors and normal tissue is hypermethylated.
The methylation-specific PCR assay involves modification of
the DNA by sodium bisulfite (converting all unmethylated but
not methylated cytosines to uracil) and subsequent
25 amplification with primers specific for methylated versus
unmethylated DNA.

Western blot analysis.   Western blot analysis of Beclin
protein may be performed on protein extracted from normal and
30 malignant breast, ovarian, and prostate tissue and cell
lines, using the polyclonal human Beclin peptide antibody
(amino acids 1-15) 843 (custom production by Eurogenetics,
Belgium) and the ECL methods according to the manufacturer's
instructions (Amersham). The amount of protein loaded may

be normalized by also probing with an antibody that reacts
with actin.

Immunocytochemical analysis. The cell type-specific
expression patterns of human Beclin in normal and malignant
breast, ovarian, and prostate tissue may be examined using
the anti-Beclin 843 antibody and the ABC immunoperoxidase
methods, according to the manufacturer's instructions (Vector
Laboratories).

**Construction of cell lines that conditionally express
wild-type and beclin mutants.** The human breast carcinoma
cell line (derived from a tumor containing 17q21 LOH) may be
used. MCF-tet off cells (Clontech) may be transfected with
wild-type *beclin* and with any mutant *beclin* cDNAs that are
detected in ovarian, breast, or prostate cancer to generate
stable MCF7 cell lines that express tetracycline-regulatable
levels of wild-type and mutant Beclin proteins. These cell
lines may be used in the tumorigenicity assays described in
the next section.

Tumorigenicity assays. Four week old female nude mice may
be injected subcutaneously (s.c.) into the left inframammary
pad with 5 x 106 tumor cells transfected with control
plasmids or plasmids containing wild-type or mutant beclin
(5 mice per treatment group) and may be implanted with a slow
release estrogen pellet (1.7 mg/60 days) in the right dorsal
thoracic region. Mice may also be implanted with either a
slow release tetracycline pellet or placebo pellet in the
left dorsal thoracic region. Tumor size may be measured
biweekly and animals may be necropsied at eight weeks after
injection for determination of tumor weight,
histopathological studies, and analysis of gene expression.

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Third Series of Experiments

The cellular antiapoptotic gene *bcl-2* represents a novel
class of antiviral host defense molecules which function both
by restricting viral replication and by preventing virus-
induced cell death. Bcl-2 blocks apoptosis in vitro induced
by several different RNA viruses, including Sindbis virus
(18, 41), influenza virus (13, 26), reovirus (31), Semliki
Forest virus (34), LaCrosse virus (29), and Japanese B
encephalitis virus (21). Previously, we have shown that Bcl-
2 overexpression in virally infected neurons in vivo also
protects mice against fatal encephalitis caused by the
prototypic alphavirus, Sindbis virus (17). The protective
effects of Bcl-2 against fatal Sindbis virus encephalitis
were associated with a reduction both in neuronal apoptotic
death and in central nervous system (CNS) viral replication.
A similar antiviral effect of Bcl-2 overexpression has been
observed during Sindbis virus infection in cultured AT3 cells
(41) as well as during influenza virus infection of MDCK
cells (26), Japanese B encephalitis virus infection of N18
cells (21), and Semliki Forest virus infection of AT3 cells
(34). Although the role of endogenous Bcl-2 in antiviral
defense has yet to be evaluated, these studies support the
hypothesis that Bcl-2 may be important in protecting cells
against viral infections.

Most previous studies examining the effects of Bcl-2 on viral
infections have been with neurotropic RNA viruses (e.g.,
Sindbis virus, reovirus, Semliki Forest virus, LaCrosse
virus, and Japanese B virus) (18, 21, 29, 31, 34, 41).
Although Bcl-2 may affect viral replication and virus-induced
apoptosis with non-neurotropic virus (e.g., influenza virus)
(13, 26), host mechanisms to inhibit apoptosis may be of
particular importance during viral infections of vital,
nonrenewable cell populations such as neurons. In such

instances, virus-induced apoptotic death of neurons may
result in irreversible CNS pathology and death of the host
organism (1, 17, 19, 25). Therefore, while apoptosis in
other cell types may be an adaptive host defense strategy
5 that reduces total viral burden for the organism (reviewed
in references 11, 16, 36, and 39), unique strategies may have
evolved to permit control of CNS viral replication without
inducing apoptotic death of infected neurons. It is thus
possible that cellular genes that play a role in preventing
10 apoptosis during normal neuronal development (e.g., bcl-2 and
bcl-x_L) (reviewed in reference 23) may also be important in
regulating CNS viral replication and in defending against
apoptosis induced by neurotropic viruses.

15 To further understand how the cellular gene bcl-2 exerts
antiapoptotic and antiviral effects during CNS viral
infection, we performed a yeast two-hybrid screen to identify
Bcl-2-interacting gene products in adult mouse brain. In
this study, we describe the identification of a novel Bcl-2-
20 interacting gene product, which we named Beclin because of
its predicted coiled-coil structure (hence, the "-in" suffix)
and its interaction with Bcl-2 (Becl). Like Bcl-2
overexpression, Beclin overexpression in neurons in vivo can
inhibit Sindbis virus replication, reduce CNS apoptosis, and
25 provide protection against fatal Sindbis virus infection.
A Beclin construct lacking the putative Bcl-2-binding domain
provides no protection and has no antiviral activity. The
findings identify a novel protein, Beclin, which may play a
role in host defense against Sindbis virus infection. In
30 addition, they suggest that interactions with Bcl-2-like
proteins may be important for the protective activity of
Beclin.

35

RESULTS

Identification of *beclin*, a novel gene on chromosome 17q21.

Previously, we demonstrated that Bcl-2 inhibits Sindbis virus
replication and prevents Sindbis virus-induced apoptosis in
5 mouse neurons (17). To further understand the mechanisms by
which Bcl-2 protects against Sindbis virus infection in
neurons, we performed a yeast two-hybrid screen of an adult
mouse brain library for complementary DNAs encoding proteins
that bind to the cell death inhibitor Bcl-2. We constructed
10 a bait plasmid (pGBT9/*bcl-2*) by fusing human *bcl-2* (lacking
the C-terminal signal-anchor sequence to ensure translocation
to the nucleus) to the GAL4 DNA-binding domain, which was
cotransformed with an oligo(dT) and random hexamer-primed
adult mouse brain cDNA fusion library in a GAL4 activation
15 domain vector, pGAD10. Of 1 million transformants, one
positive colony was identified by the 5-bromo-4-chloro-3-
indolyl-β-D-galactopyranoside   (X-Gal)   filter   assay.
Sequencing analysis of the cDNA plasmid rescued from this
colony (F1) revealed a termination condon 42 bp downstream
20 from the GAL4 activation domain, several predicted short open
reading frames between nucleotides 124 and 1843 and a longer
predicted open reading frame (with a good Kozak consensus
sequence and multiple stop codons upstream) spanning from
nucleotide 1855 to the 3' end of the insert. Thus, either
25 the 14-aa fusion protein was interacting with Bcl-2 or one
of the downstream open reading frames encoded a protein that
contains its own activation domain and interacts with Bcl-2.
To identify the Bcl-2-interacting region of F1, we fused
nucleotides 1 to 1854 and 1855 to 2500 to the GAL4 activation
30 domain in pGAD424 and tested for interactions with Bcl-2.
Nucleotides 1855 to 2500, but not 1 to 1854, encoded a
protein that interacts with Bcl-2 fused to the GAL4 DNA-
binding domain (Table 1) but not with control GAL4 (DNA-
binding domain plasmids containing p53, lamin (Table 1), or
35 Sindbis virus glycoproteins (data not shown).

Table 1.   Summary of yeast two-hybrid assay results

GAL4 activation domain plasmid[b]	β-Galactosidase reaction with GAL4 binding domain construct[a]						
	Empty	Bcl-2	Bcl-x$_1$	Bcl-x$_s$	Bax	Lamin	p53
Empty	−	−	−	+	.−	−	−
F1							
1-2563	−	+	+	ND	−	−	−
1-1855	−	−	−	ND	−	−	−
1856-2563 (Mus Beclin 1-708)	−	+	+	ND	−	−	−
Human Beclin							
1-708	−	+	+	ND	−	−	−
Beclin 1-450	−	+	+	ND	−	−	−
Beclin 1-258	−	−	−	ND	−	−	−
Beclin 262-450	−	+	+	ND	−	−	−
Beclin 451-708	−	−	−	ND	−	−	−
Beclin 1-1353	−	−	−	ND	−	−	−

[a]+, positive reaction within 15 min; −, lack of positive reaction at 24 h; ND, not determined.
[b] Nucleotide positions of genes fused to the plasmid.

A database search revealed that the nucleotide sequence of F1 (1855 to 2500) overlapped with sequences of several clones isolated from a normalized infant human brain cDNA library in the Merck EST (epitope tag sequence) database as well as clones from human breast (GT197) (32) and human fibroblast (B32) cells (8). These clones contain only partial open reading frames of a novel gene that encodes a protein with coiled coils. As explained above, we assigned the name *beclin* to this gene because of the interaction of its encoded protein with Bcl-2 and the predicted coiled-coil structure of its encoded protein. Clones GT197 and B32 were both isolated in the generation of transcription maps of the breast cancer susceptibility locus on chromosome 17q21 and are mapped to a region located approximately 100 kb centromeric to the gene *BRCA1*. They lie within a 400-kb minimal deletion unit mapped by Tangir et al. in sporadic epithelial ovarian cancers (38).

We aligned the overlapping partial clones in GenBank with mouse *beclin* sequence to obtain a predicted sequence of the

full-length open reading frame of human *beclin* and isolated
human *beclin* from a normalized human infant brain cDNA
library (37). Human *beclin* is predicted to encode a novel
450-aa, 60-kDa protein containing a coiled-coil region with
5   25 to 28% homology with myosin-like proteins (Fig.13). It
shares 93% identity at the nucleotide level and 98% identity
at the amino acid level with the mouse *beclin* sequence
identified in the yeast two-hybrid screen. Human Beclin is
also homologous with the *Ceanorhabditis elegans* T19E7.3 gene
10 product (GenBank accession no. U42843) and the *S. cerevisiae*
gene product Lph7p (GenBank accession no. U43503) (38 and 37%
identical over 145 and 137 residues, respectively),
indicating a high degree of evolutionary conservation.
PROSITE analysis of human Beclin identified several potential
15 glycosylation, phosphorylation, and myristoylation sites but
no other functional sequence motifs.

**Ubiquitous expression of *beclin* mRNA in mouse and human
tissues.** To examine the tissue-specific pattern of *beclin*
20 expression, we hybridized mouse and human multiple-tissue
Northern blots with a *beclin*-specific probe. RNA blot
analysis revealed that expression of *beclin* mRNA is
widespread in both mouse and human adult tissue (Fig. 14A).
A *beclin*-specific probe hybridized to a 2.2-kb transcript
25 present at highest levels in human skeletal muscle but at
detectable levels in all tissues examined. The size of this
transcript is approximately the same as that observed
previously for clones GT197 and B32 (8, 32). In some
tissues, additional 1.7- and 1.4-kb transcripts were
30 observed, suggesting the presence of alternatively spliced
transcripts.

Beclin is expressed in human neurons. To examine whether
Beclin protein is expressed in neurons (the primary CNS
35 target cell type for Sindbis virus infection), we performed

immuno-peroxidase staining of adult human brain sections.
We used rabbit immune serum 843, generated against a human
Beclin peptide corresponding to aa 1 to 15. 843 reacts with
a 61-kDa protein in lysate prepared from BHK cells after
5 infection with a recombinant Sindbis virus containing a flag
epitope-tagged human *beclin* insert (SIN/*beclin*) (Fig. 15B).
This protein migrates identically to the major band detected
with an anti-flag epitope antibody in SIN/*beclin*-infected BHK
cell lysate (Fig. 15A) and to in vitro translated flag-Beclin
10 (data not shown). Immunoperoxidase staining of human brain
sections from the hippocampus and frontal cortex revealed
Beclin immunoreactivity in many neurons throughout these
regions as well as in some glial cells. In neurons, Beclin
demonstrated a granular, punctate pattern of staining that
15 was found almost exclusively in the region of the perikaryon
(Fig. 14B). Beclin immunoreactivity was also observed in the
media of blood vessels, in the ependymal cells, and in the
choroid plexus. No staining was observed in human brains
stained with rabbit preimmune serum 843 (data not shown).
20

Interaction of Beclin and Bcl-2. We performed additional
yeast two-hybrid studies to confirm that human *beclin*, like
mouse *beclin*, encodes a protein that interacts with human
Bcl-2 and to further define the Bcl-2-interacting region of
25 human Beclin (Table 1). We found that the region of human
Beclin that corresponds to the mouse gene product isolated
in the yeast two-hybrid screen (aa 1 to 236) also interacts
with Bcl-2. Further deletion mutation analysis revealed that
aa 88 to 150 of Beclin were sufficient to mediate an
30 interaction with Bcl-2. Interestingly, the coding sequence
for this region of Beclin is deleted in some human infant
brain cDNA clones in the Merck EST database, suggesting that
Beclin exists in at least two forms, including one that
contains a Bcl-2-binding domain and one that lacks this
35 domain. Full length-Beclin does not interact with Bcl-2 in

the yeast two-hybrid system. As noted below, when expressed
in transient transfection assays, flag-tagged full-length
human Beclin displays a punctate immuno-reactivity pattern
suggestive of association with intracellular organelles and
5   is associated with the insoluble fraction after cell lysis.
In contrast, a flag-tagged truncated Beclin (aa 1 to 236)
(corresponding to the region isolated in the yeast two-hybrid
screen) displays a diffuse cytoplasmic staining pattern and
is soluble after cell lysis. These differences between full-
10   length and truncated Beclin are thought to account for
differences in ability to translocate to yeast nuclei and
interact with Bcl-2 in the yeast two-hybrid assay.

To directly examine whether full-length human Beclin and Bcl-
15   2 interact in mammalian cells, we performed FRET studies of
COS7 cells cotransfected with Bcl-2 and flag epitope-tagged
Beclin. Beclin is predicted to be a coiled-coil protein that
may be associated with the cytoskeleton, and it partitions
with the insoluble fraction following cell lysis. For this
20   technical   reason,   biochemical   analyses   of in   vivo
interactions between Bcl-2 and Beclin cannot be performed.
FRET is a fluorescence technique that can be used as a
spectroscopic ruler to study and quantify the interactions
of cellular components with each other (reviewed in
25   references 6, 12, and 35). In FRET, a fluorophore (donor)
in an excited state may transfer its excitation energy to a
neighboring chromophore (acceptor) nonradiatively through
dipole-dipole interactions. The efficiency of this process
varies as the inverse of the sixth power of the distance
30   separating the donor and acceptor chromophores and, in
practice, requires the distance between the donor and
acceptor fluorophores to be short (usually less than 50 Å).
The dependence of the energy transfer efficiency on the
donor-acceptor separation provides the basis for the utility
35   of this phenomenon in the study of cell component
interactions.   FRET has been used by a number of

investigators   to   examine   interactions   of   cellular
constituents (reviewed in 6, 12 and 35) such as endosomal
fusion events, ligand-dependent growth factor receptor
aggregations, interactions of viral and cellular proteins
with regulators of apoptosis (20), and interactions of
cellular cytoskeletal components (33).

Prior   to   measuring   FRET,   we   first   confirmed   the
colocalization of full-length Bcl-2 and Beclin in transfected
COS7 cells, using confocal laser microscopy (Fig. 16). Bcl-2
is known to associate with the outer mitochondrial membrane,
endoplasmic reticulum, and perinuclear membranes, and it
displays a punctate pattern of cytoplasmic immunoreactivity
(reviewed in reference 28).   We found that flag-Beclin
invariably displayed a pattern of immunoreactivity identical
to that of Bcl-2 in all cotransfected COS7 cells examined by
confocal laser microscopic analysis (Fig. 16A to C).
Furthermore, we found that deletion of the putative Bcl-2-
binding domain from Beclin did not alter its pattern of
immunoreactivity; flag-BeclinΔBcl-2BD appeared to have a
pattern of staining similar to that of full-length Beclin,
and like full-length Beclin, it colocalized with Bcl-2 in
cotransfected cells (Fig. 16D to F). This granular pattern
to Beclin immunoreactivity in the perinuclear region is
similar to that observed for endogenous Beclin in human
neurons (compare Fig. 14B, 16A and 16D).

After confirming the colocalization of Bcl-2 and Beclin and
of Bcl-2 and BeclinΔBcl-2BD, we used FRET analysis to
determine whether BCL-2 and Beclin physically interact. We
compared in transfected COS7 cells the amounts of FRET
between Bcl-2 and full-length Beclin, Bcl-2 and BeclinΔBcl-
2BD, and Bcl-2 and a control protein, SERCA. Quantitative
analysis of microscopic images (following corrections for
cross-talk between filter sets and donor and acceptor
concentrations showed significantly more FRET in cells with

labeled full-length Beclin and Bcl-2 ($E_{Beclin-Bcl-2}$= 0.000578 ±
0.000262; mean ± standard error of the mean [SEM], n = 410)
than in cells with labeled BeclinΔBcl-2BD and Bcl-2 ($E_{BeclinΔBcl-2BD-Bcl-2}$ = 0.000189 ± 0.000151; n = 2,946) (P = 0.0068, t test)
or in cells with labeled SERCA and Bcl-2 ($E_{SERCA-Bcl-2}$ =
0.0000639 ± 0.0000390; n = 775) (P = 0.0021, t test).
These quantitative analyses indicate that Beclin and Bcl-2
exhibit FRET and provide evidence of an interaction between
these two proteins in mammalian cells. Furthermore, deletion
of the Bcl-2-binding domain of Beclin mapped in yeast two-
hybrid studies does not alter the spatial orientation of
transfected Beclin, but it does significantly decrease FRET.
This observation suggests that the FRET observed between
full-length Beclin and Bcl-2 reflects a specific association
of these proteins in vivo, rather than an artifact secondary
to overexpression.

**Selective interaction of Beclin with death repressor members
of the Bcl-2 family.** To investigate whether Beclin interacts
with other Bcl-2 family members that positively or negatively
regulate apoptosis, we fused *bax*, *bcl-x$_S$*, and *bcl-x$_L$* into the
GAL4 binding domain vector and tested for interactions with
Beclin in the yeast two-hybrid system (Table 1). The Bcl-x$_s$
GAL4 binding domain construct activated transcription by
itself and therefore could not be tested for interactions
with Beclin. The same region of Beclin (aa 88 to 150) that
interacted with Bcl-2 also interacted with Bcl-x$_L$, a related
Bcl-2 family member that inhibits apoptosis (4). In
contrast, Beclin did not react with Bax, a family member that
promotes apoptosis (27). This pattern of interaction, i.e.,
with Bcl-2 and Bcl-x$_L$, but not Bax, is identical to that
observed for all previously identified Bcl-2-interacting
proteins outside the Bcl-2 family (reviewed in reference 30).

**Mutations in Bcl-2 and Bcl-x$_L$ that block death repressor
activity also block binding to Beclin.** Cheng et al. have
shown that Bcl-2 and Bcl-x$_L$ overexpression can delay Sindbis
virus-induced death of BHK cells (5). To evaluate whether
5   Bcl-2-Beclin and Bcl-x$_L$-Beclin interactions may be related to
this ability of Bcl-2 and Bcl-x$_L$ to inhibit Sindbis virus-
induced apoptosis, we constructed pGBT9 vectors containing
bcl-2 and bcl-x$_L$ constructs with mutations in the conserved
BH1 domain that are known to block death repressor activity.
10 A Gly-Ala mutation at amino acid position 145 of Bcl-2
completely abrogates Bcl-2 death repressor activity in
interleukin-3   deprivation-,   γ-irradiation-   and
glucocorticoid-induced apoptosis (42) and also blocks Bcl-2
binding to Beclin in the yeast two-hybrid system (Table 2).
15 Similarly, substitution of aa 136 to 138 of Bcl-x$_L$ (VNX→AIL)
completely abolishes death repressor activity in Sindbis
virus-induced apoptosis (5) and also blocks Bcl-x$_L$ binding to
Beclin (Table 2). These mutations in Bcl-2 (G→A145) and
Bcl-x$_L$ (VNW→AIL) did not alter the level of Bcl-2 or Bcl-x$_L$
20 expression in yeast cells (data not shown), indicating that
the lack of interaction could not be attributed to effects
of the mutations on protein expression in yeast. These data
therefore demonstrate that mutations that block the antideath
activity of Bcl-2 and Bcl-x$_L$ also block binding to Beclin .

TABLE 2.   Effect of BH1 domain mutations on the ability of
           Bcl-2 and Bcl-x$_L$ to bind to Beclin in the yeast
           two-hybrid assay

Construct[a]	Conserved BH1 domain	Inhibition of apoptosis (reference)	Beclin binding
WT Bcl-2	ELFRDGVNWGRIVAFFEFGG	+	+
WT Bcl-x$_L$	ELFRDGVNWGRIVAFFSFGG	+	+
MT Bcl-2	--------A---------	- (42)	-
MT Bcl-x$_L$	--------AIL-------	- (5)	-

[a]WT, wild-type; MT, mutant.

**Beclin protects mice against fatal encephalitis caused by the
neurovirulent TE12 strain of Sindbis virus.**   After
identifying Beclin as a novel Bcl-2-interacting protein that
is expressed in neurons, we were interested in studying its
effects on Sindbis virus infection. To directly evaluate the
role of Beclin and its potential interactions with Bcl-2-like
proteins in modulating Sindbis virus pathogenesis, we
compared the natural histories of mice infected with
recombinant chimeric viruses that express either wild-type
human Beclin (SIN/beclin), human Beclin lacking the putative
Bcl-2-binding domain (SIN/beclinΔ Bcl-2BD), or human Beclin
containing a premature stop codon at nucleotide position 270
(SIN/beclinstop).

To construct these viruses, we used a strategy identical to
that previously described for Sindbis virus chimeras that
express human Bcl-2 (17), with the exception that we used a
more neurovirulent background strain of Sindbis virus,
dsTE12. We chose dsTE12 rather than the previously used less
virulent strain ds633 because of the possibility that the
larger size of the human beclin open reading frame insert
would have an attenuating effect on Sindbis virus
neurovirulence.   Whereas ds633 is neurovirulent only in
neonatal mice, dsTE is also neurovirulent in older mice (40).
ds633 and dsTE12 differ at one amino acid position in the E2
envelope glycoprotein (Gln-55 in ds633; His-55 in dsTE12) and
two amino acid positions in the E1 glycoprotein (Val-72 and

Gly-313 in ds633; Ala-72 and Asp-313 in dsTE12). Previous
studies with recombinant viruses have mapped the amino acid
residue responsible for neurovirulence in older mice to
position 55 in E2 (40, 41).

5

We confirmed that SIN/*beclin* and SIN/*beclin*ΔBcl-2BD expressed
proteins of the predicted molecular weights by performing
Western blot analyses of infected BHK cell lysates with both
an anti-flag epitope monoclonal antibody and a polyclonal
10 anti-human Beclin peptide antibody (Fig. 15A and B).
SIN/*beclin* expressed a 61-kDa protein and SIN/*beclin*ΔBcl-2BD
expressed a 52-kDa protein reactive with both anti-flag
epitope and anti-Beclin antibodies. We also confirmed that
human flag-Beclin and flag-BeclinΔBcl-2BD were expressed in
15 virally infected neurons by performing immunoperoxidase
staining of mouse brains at days 1, 2, and 4 after infection
(representative photomicrographs shown in Fig. 15C and E).
Although we did not detect any flag-Beclin protein expression
by Western blot analysis of BHK cell lysates infected with
20 SIN/*beclin*stop, flag immunoreactivity was also observed in
mouse brains infected with SIN/*beclin* (Fig. 15D). No flag
immunoreactivity was seen in control mouse brains infected
with the dsTE12 vector alone (data not shown). Fewer flag-
immunoreactive cells were observed in SIN/*beclin*-infected
25 than in SIN/*beclin*ΔBcl-2- and SIN/*beclin*stop-infected mouse
brains.

We compared the survival of 10-day-old litters of CD1 mice
infected intracerebrally with 1,000 PFU of SIN/*beclin*,
30 SIN/*beclin*ΔBcl-2BD, and SIN/*beclin*stop (Fig. 17A). The
survival of mice infected with SIN/*beclin* was 71%, compared
with only 9 and 7% after infection with SIN/beclinΔBcl-2BD
and SIN/*beclin*stop. The differences in survival between
groups infected with SIN/*beclin* versus SIN/*beclin*ΔBcl-2BD and
35 Sin/*beclin* versus Sin/*beclin*stop were highly significant

(P<0.001; life-table analysis). Thus, full-length Beclin
overexpression in virally infected neural cells results in
marked protection against fatal Sindbis virus encephalitis.
Furthermore, the high mortality after infection with a virus
5   expressing a mutant form of Beclin lacking the Bcl-2 binding
domain suggests that binding to Bcl-2 or Bcl-2-like proteins
is important for the protective effects of Beclin on survival
in Sindbis virus encephalitis

10   **Bcl-2 does not protect against fatal encephalitis caused by**
TE12.   Previously, we showed that Bcl-2, expressed in the
ds633 Sindbis virus vector, protected neonatal mice against
fatal Sindbis virus infection (17). However, in in vitro
studies in AT3/bcl-2 cells, the TE12 strain overcomes
15   protection conferred by Bcl-2 (41). To directly compare the
abilities of Beclin and Bcl-2 to protect against encephalitis
by TE12 in 10-day-old mice, we cloned human *bcl-2* and human
bcl-2 containing a premature stop codon after nucleotide
position 118 into the dsTE12 vector to generate the
20   constructs SIN/*bcl-2* and SIN/*bcl-2*stop. Unlike Sin/*beclin*
versus SIN/*beclin*stop (Fig. 17A), there was no difference in
the survival of mice infected with SIN/*bcl-2* versus SIN/*bcl-*
*2*stop (Fig. 17B). Together with previous results (17), these
data demonstrate that Bcl-2 can protect against fatal
25   encephalitis caused by a strain of Sindbis virus containing
a wild-type glutamine at position E2 position 55 but not by
a more neurovirulent strain of Sindbis virus containing a
histidine mutation at E2 position 55 (E2-55 histidine
mutation).

30
Beclin reduces Sindbis virus replication in mouse brain. To
examine whether Beclin overexpression affects Sindbis virus
replication, we measured viral titers of mouse brain
homogenates and performed in situ hybridization of mouse
35   brain sections to detect message-sense viral RNA. Mean viral

titers were reduced 5-fold at day 1 and 50-fold at days 2 and
4 after infection in brains of mice infected with Sin/*beclin*
compared to the brains of mice infected with SIN/*beclin*stop
or SIN/*beclin*Δbcl-2BD (Fig. 18). Computerized quantitative
5   image analysis of virus RNA positive cells in infected mouse
brains showed an inhibitory effect of Beclin on viral
replication that was similar to, but had a temporal pattern
somewhat different from, that observed by measurement of
viral titers (Fig. 19). The mean number of virus RNA-
10 positive cells was significantly lower in brains infected
with SIN/*beclin* than in brains infected with SIN/*beclin*stop
at day 4 after infection (100 ± 36 versus 494 ± 55; P =
0.004, t test) but was equivalent at day 2 after infection
(575 ± 272 versus 507 ± 216; P = 0.854, t test). The
15 reduction of viral titers but not of virus RNA-positive cells
in mouse brains 2 days after infection with SIN/*beclin*
suggests a possible inhibitory effect of Beclin on
posttranscriptional stages of viral replication. In
addition, the viral titers of SIN/*beclin*ΔBcl-2BD-infected
20 mouse brains did not differ significantly from titers of
SIN/*beclin*stop-infected mouse brains, but the number of virus
RNA-positive cells was higher at days 2 and 4 in
SIN/*beclin*ΔBcl-2BD-infected than in SIN/*beclin*stop-infected
mouse brains. This difference was significant at day 4
25 (905±72 versus 494 ± 55; P = 0.01, t test) but not a day 2
(1,190 ± 375 versus .507 ± 216; P = 0.190, t test) after
infection.

The anatomic distribution of RNA-positive cells did not
30 differ among the difference virus-infected groups; viral RNA-
positive cells were found in clusters scattered throughout
the brain, most commonly in the subventricular zone,
posterior neo-cortex, colliculus, hippocampus, striatum, and
olfactory bulb. However, the number of cells seen within a
35 given cluster was usually lowest in SIN/*beclin* Δ Bcl-2BD-

infected mouse brains (see representative photomicrographs
in Fig. 21).

**Beclin reduces Sindbis virus-induced cell death in mouse
brain.** To examine whether CNS Beclin overexpression reduces
apoptotic cell death, we quantitated the number of apoptotic
nuclei in the brains of mice infected with the chimeric
viruses SIN/*beclin*, SIN/*beclin*stop, and SIN/*beclin* Δ Bcl-2BD
(Fig. 20). Within each mouse brain, almost all apoptotic
nuclei were found in regions that had detectable viral RNA
by in situ hybridization (Fig. 21) and in regions that had
histopathologic evidence of neuronal death. A close
asociation was observed between the mean numbers of apoptotic
nuclei at each time point among hte three different virus
treatment grops and the mean numbers of virus RNA-positive
cells (compare results in Fig. 19 and 20). In mouse brains
infected with SIN/*beclin*stop, little cell death was seen at
days 1 and 2 after infection, and the number of apoptotic
cells per square millimeter of brain did not differ between
these groups. More apoptotic nuclei were present at day 2
after infection in the brains of mice infected with
SIN/*beclin* Δ Bcl-2BD (604 ± 211) than in the brains of mice
infected with SIN/*beclin* (133 ± 90) or SIN/*beclin*stop (91 ±
52); this deifference was nearly statistically significant
($P = 0.078$, analysis of variance). At day 4 after infection,
the mean number of apoptotic cells per square millimeter of
brain was markedly increased in SIN/*beclin*stop (1,490 ± 29)
and SIN/*beclin* Δ Bcl-2BD (1,044 ± 430) versus SIN/*beclin*-
infected mouse brains (101 ± 10; $P = 0.001$, analysis of
variance). These data demonstrate that Beclin overexpressio
ndecreases apoptotic death in mouse brains infected with TE12
strain of Sindbis virus.

DISCUSSION

In this study, we describe the identification of a novel coiled-coil 60-kDa protein, Beclin, which interacts with the cell death regulator Bcl-2. We demonstrate that Beclin overexpression in virally infected neurons in vivo results in substantial protection against Sindbis virus-induced disease. Mice infected with recombinant Sindbis viruses that express wild-type Beclin have less neural cell apoptosis, decreased viral replication, ad a significant lower mortality rate than mice infected with recombinant viruses that express Beclin deletion mutants. Furthermore, the Bcl-2 binding domain of Beclin is required for the antiviral, antiapoptotic, and survival-promoting effects of Beclin on CNS Sindbis virus infection. These findings suggest that Beclin, via interactions with Bcl-2-like molecules, can function in vivo in the CNS as an antiviral host defense molecule.

The protective, antiviral effects of Beclin are mediated through inteactions with Bcl-2 (or Bcl-2-like molecules that bind to same region of Beclin as Bcl-2) is supported by data obtained with a chimeric Sindbis virus expressing Beclin lacking aa 88 to 151. Amino acids 88 to 150 of Beclin are sufficient to mediate an interaction with Bcl-2 and Bcl-x_1 in the yeast two-hybrid assay, and deletion of these amino acids from full-length Beclin significantly decreases the strength of the Beclin-Bcl-2 interaction in mammalian cells. The Sindbis virus construct expressing Beclin lacking aa 88 to 150 (SIN/beclinΔBcl-2BD) replicated to as high levels in muse brain, resulted in as much apoptotic neural cell death, and resulted in the same mortality in mice as a control recombinant virus which expressed a 90-aa truncated Beclin protein (SIN/beclin-stop). The lack of protedtive activity of BeclinΔBcl-2BD compared to full-lenght Beclin cannot be

attributed to lower levels of BeclinΔBcl-2BD expression after
infection with SIN/*beclin*ΔBcl-2BD. The levels of flag-
BeclinΔBcl-2BD were equal to or greater than levels of flag-
Beclin detected immunoblot analysis of lysates from BHK cells
5   infected   with   SIN/*beclin*   and   SIN/*beclin*ΔBcl-2BD,
respectively. In addition, SIN/*beclin*ΔBcl-2BD-infected mouse
brains had more flag-immunoreactive cells than mouse brains
infected with SIN/*beclin.*

10 Thus, the lack of protective activity of BeclinΔBcl-2 is most
consistent with the hypothesis that the antiviral effects of
Beclin are mediated through interactions with Bcl-2-like
proteins.

15 Some of the data in the present study suggest that the virus
SIN/beclinΔBcl-2BD may be more neurovirulent than the virus
SIN/*beclin*stop. Mice infected with SIN/*beclin*ΔBcl-2BD had
more virus RNA-positive cells in their brains in days 2 and
4 after infection than mice infected with compared to
20 SIN/*beclin*stop. Although the overall mortality rates of mice
infected with SIN/*beclin*ΔBcl-2BD versus SIN/*beclin*stop did
not differ, but the mean day of death was slightly lower in
mice infected with SIN/*beclin*ΔBcl-2BD versus SIN/*beclin*stop.
These observations raise the possibility that Beclin lacking
25 the Bcl-2-binding domain functions as a dominant negative
mutant.   As a corollary, the naturally occurring Beclin
isoform found in brain that lacks the Bcl-2-binding domain
may serve as a death-promoting molecule.

30 Beclin may exert antiviral effects via interactions with
either Bcl-2, Bcl-x_1, or yet unidentified Bcl-2 family
members. In yeast two-hybrid assays, we found that the same
region of Beclin interacted with Bcl-2 and Bcl-x_1, and that
loss-of-function mutations in the conserved BH1 domains of
35 both Bcl-2 and Bcl-x_1, blocked binding to Beclin. While both

Bcl-2 and Bcl-x_1, are important regulators of apoptosis in
neurons (reviewed in reference 23), we have found that Bcl-2,
but not Bcl-x_1, protects mice against fatal encephalitis and
delays Sindbis virus-induced death of cultured rat dorsal
5   root ganglion neurons (14,17). Thus, Bcl-2 may be more
important than Bcl-x_1, as a regulator of Sindbis virus-
induced death in neural cells. Additional studies examining
the effects of Beclin on Sindbis virus infection in mice that
are deficient in Bcl-2 may help define whether Bcl-2 is the
10 biologically important Hbeclin-binding partner in in vivo
antiviral pathways in the CNS.

The mechanisms by which Beclin, in cooperation with Bcl-2-
like proteins, functions to inhibit Sindbis virus replication
15 and sindbis virus-induced neuronal death are unknown. Both
Simliki Forest virus and Sindbis virus have been shown to
inactivate the antiapoptotic function of Bcl-2 in transfected
fibroblasts through capase-induced Bcl-2 clevage (10). In
addition, Nava et al. have shown that crmA increases the
20 survival of Sindbis virus-infected mice (24), suggesting a
role for fatal encephalitis. One possibility, therefore,
is that binding to Beclin somehow protects Bcl-2 (or Bcl-x_1)
from clevage by caspases, thereby preventing Sindbis virus-
induced cell death. A second possibility is that the Beclin-
25 Bcl-2-like protein complex blocks an endoplasmic reticulum
stress signal triggered by the Sindbis virus E2 and E1
envelope glycoproteins. One of the mechanisms by which Bcl-2
and Bcl-may inhibit apoptosis is by regulating the
permeability of intracellular membranes (reviewed in
30 reference 30). Recently, we have shown that the
overexpression of the transmembrane domains of the Sindbis
virus E2 and E1 envelope glycoproteins induces apoptosis in
AT3 cells (15). In addition, coiled-coil proteins such as
Beclin may play a role in linking membrane signal trasduction
35 events with the cytoskeleton. Thus, we speculate that
Beclin, a protein which localizes to intracellular membranes,

may be part of a complex with Bcl-2-like proteins that
regulates signalling events initiated by Sindbis virus
envelope glycoproteins inthe endoplasmic reticulum.

5   Many similarities exist between the findings of the present
study and work examining the effects of Bcl-2 overexpression
on the natural history of Sindbis virus encephalitis. As
observed with Beclin in this study, the overexpression of
Bcl-2 in virally infected neurons has previously been shown
10 to   reduce   CNS   apoptosis,   reduce   CNS   Sindbis   virus
replication, and reduce Sindbis virus mortality (17).
However, in the present study, we also identify one
significant biologic difference between Bcl-2 and Beclin;
namely, Beclin can exert protective effects against infection
15 by a neurovirulent strain of Sindbis virus that is known to
overcome the protective effects of Bcl-2. Ubol et al.
demonstrated that a histidine substitution for wild-type
glutamine at position 55 of the Sindbis virus E2 envelope
glycoprotein was sufficient to enable the virus to kill cells
20 expressing Bcl-2(41). Similarly, we have previously found
that Bcl-2 expressed in a strain of Sindbis containing a
glutamine at position 55 (strain 633) protects against fatal
encephalitis (17), but in the present study, we found that
Bcl-2 expressed in a strain of Sindbis containing a histidine
25 at position 55 (strain TE12) confers no protection against
fatal disease. Yet, when expressed in an E2-55 histidine-
containing background strain of Sindbis virus, Beclin confers
significant protection. Thus, the mutation in E2, which
confers neurovirulence, can counter the protective effects
30 of Bcl-2 but not of Beclin.

Several explanations have been proposed to explain the
ability of the E2-55 histidine mutation to confer resistance
to Bcl-2 protection agaisnt Sindbis virus-induced death.
35 Ubol et al. suggested that the neurovirulent mutation in E2
might somehow alter either a direct interaction between E2

and Bcl-2 in the endoplasmic reticulum or a possible effect
of Bcl-2 on E2 protein folding or posttranslational
modifications (41). More recently, Grandgirard et al. raised
the possibility that the mutation in E2 facilitates the
access of caspases to Bcl-2 and subsequent triggering of Bcl-
2 cleavage(10). If viral activation of caspase-induced
cleavage of Bcl-2 does prove to be an important mechanism of
neurovirulence of Sindbis virus strains containing E2-55
histidine, Beclin may serve as an important host defense
factor against this strategy of neurovirulence. This
hypothesis is based on the ability of Beclin, a Bcl-2
interacting protein, to protect against neuronal death
induced by the TE12 strain of Sindbis virus.

The antiviral effects of Beclin observed in Sindbis virus-
infected mouse brains may be exerted at a stage of viral
replication after viral RNA synthesis. At 2 days after
chimeric virus infection, Beclin overexpression was
associated with a 50-fold reduction in viral titers but no
reduction in the number of viral RNA-positive cells. This
finding suggests a block or abnormality at the level of
either translation of the Sindbis virus structural proteins,
posttranslational modifications of viral glycoproteins, virus
assembly, or budding. This observation contrasts with
previous studies examining the effects of Bcl-2 on
infectionwith a related alphavirus, Semliki Forest virus
(34). On the basis of in situ hybridization studies for
viral RNA and immunostaining for viral proteins, Scallan et
al. concluded that *bcl-2* functions at an early stage of the
virus life cycle, either entry, pretranscriptional events,
or transcription, to inhibit Semliki Forest virus replication
(34). The discrepancy between the findings of Scallan et al.
and those reported in the present study may reflect
fundamental differences between the antiviral effects of
Beclin and Bcl-2, differences between Sindbis virus and
Semliki Forest virus, or differences between the cell types

infected.   Alternatively, it is possible that Bcl-2 and/or
Beclin act at multiple overlapping stages of the alphavirus
life cycle and that different experimental designs in the two
studies uncovered effects on different phases of replication.
5 Given the lack of antiviral activity of Baclin lackin the
Bcl-2 binding domain, it seems likely that the mechanisms by
which Beclin and Bcl-2 inhibit Sindbis virus replication in
mouse brain are similar.

10 The results of the present study do not permit us to evaluate
whether the benefical effects of Beclin on Sindbis virus-
induced mortality are a consequence of antiviral effects,
antiapoptotic effects, or a combination of both.   It is not
known whether Beclin prevents neural cell death solely as a
15 consequence of reducing Sindbis virus replicaiton in neurons
or whether Beclin exerts antiapoptotic effects independently
of its effects on viral replication.   Further studies
examining the effects of Beclin on apoptotic death in
response to nonviral stimuli will be necessary to determine
20 whether Beclin, like Bcl-2, functions as a general apoptosis
inhibitor. If so, structure-function analyses of Beclin may
be helpful to map domains important for antiviral and
antiapoptotic function and to identify whether these two
properties are interrelated or independent.
25

Beclin has antiviral activity and that Beclin interacts with
the cell death regulator.   In dividing cells, inhibition of
virus-induced cell death by virally encoded cell death
inhibitors such as p35 (7) and adenovirus E1B can increase
30 viral replication (3).   However, in nondividing, terminally
diffrentiated cells such as neurons, genetic pathways may
exist which permit preservation of the life of the cell,
without the adverse consequence of increased total viral
burden for the organism. Genes such as bcl-2 and beclin that
35 are normally expressed in mammilian neurons may be important
components of such pathways.

MATERIALS AND METHODS

Plasmids:

To construct vectors for transient expression in mammalian
5   cells, the open reading frame of human *bcl-2*, flag epitope-
tagged human *beclin*, and flag epitope-tagged *beclin* deleted
of nucleotides 238 to 453 were cloned into pSG5 (Stratagene).
To construct viral cDNA clones, the previously described
neurovirulent double subgenomic Sindbis virus vector dsTE12
10   was used. Full-length flag epitope-tagged human *beclin*, human
beclin containing a stop codon inserted at nucleotide
position 270, human *beclin* containing an in-frame deletion
of nucleotides 238 to 453, human *bcl-2*, and human *bcl-2*
containing a stop codon at nucleotide position 118 were
15   cloned into the *BstEII* site of dsTE12 to generate plasmids
SIN/*beclin*, SIN/*beclin*stop, SIN/*beclin* Δbcl-2BD, SIN/*bcl-2*,
and *SIN*/bcl-2*stop*, respectively. To construct vectors for
yeast two-hybrid studies, the sequences encoding amino acids
(aa) 1 to 218 of human *bcl-2*, 1 to 212 of *bcl-x$_L$*, 1 to 149 of
20   bcl-x $_s$ and 1 to 171 of *bax* were cloned into pGBT9 in frame
with the GAL4 DNA-binding domain. To avoid difficulties with
targeting of proteins to the nucleus, the sequences encoding
C-terminal transmembrane domains of *bcl-2* family members were
omitted. Control pGBT9 plasmids containing lamin (pLAM5')
25   and p53 (pVA3) inserts were obtained from Clontech.

Yeast Two-Hybrid Screen:

Saccharomyces cerevisiae SFY526 cells were cotransformed with
pGBT9/*bcl-2* an 10^6 cDNA molecules from an adult mouse brain
30   library   fused   to   a   GAL4   activation   domain   vector
(pGAD10:Clontech), plated onto SD medium lacking tryptophan
and leucine, incubated at 30°C for 4 days, and then screened
for LacZ activity by a colony lift filter assay. Putative
interacting clones were isolated by manipulation in *leuB*
35   *Escherichia coli*, and further tested against pGBT9 and

control plasmids.   A positive β-galactosidase reaction
between pGBT9/*bcl-2* and clone F1 was obtained within 10 to
15 min. For analysis of interactions between Beclin and Bcl-
2 family members, pGBT9 plasmids containing *bcl-2* family

5   members were cotransformed with fragments of human *beclin* (1
to 450, 262 to 450, and 1 to 708) fused to the GAL4
activation domain in pGAD424, and transformants were screened
for LacZ activity.

10   **Sequencing And Analysis Of Human Beclin:**
The partial nucleotide sequence of mouse *beclin* obtained from
sequencing clone F1 was aligned with an overlapping clone
GT197 isolated from human breast.   Primers immediately
upstream and downstream of the predicted open reading frame

15   were used to amplify the coding sequence of human *beclin* from
a normalized human infant brain cDNA library. The resulting
PCR products from several independent reactions were cloned
into pCRII and sequenced in both directions, using Sequenase
(U.S. Biochemical) as well as automated sequencing.   The

20   resulting nucleotide sequence and deduced amino acid
sequences were used to scan various data banks (GenGank,
EMBL, SwissProt, and PIR) for homologous sequences, using the
BLAST algorithms. The amino acid sequence was also analyzed
by the PROSITE program to identify functional motifs and by

25   the COILS program to identify coiled-coil regions.

Northern Blot Analysis:

Human and mouse multiple tissue blots (Clontech) were hybridized with ^{32}P randomly labeled human or mouse *beclin* probes (nucleotides 1 to 485) as instructed by the manufacturer (Clontech). Equal loading [2μg of poly(A) RNA] was confirmed by hybridization to a β-actin probe.

Production of Recombinant viruses:

The viruses SIN/*beclin*, SIN/*beclin*stop, SIN/*beclin*ΔBcl-2BD, SIN/*bcl-2*, and SIN/*bcl-2*stop were generated from viral cDNA clones as previously described. Briefly, 5'-capped transcripts were synthesized from cDNA clones linearized with *Pvu*I (for *beclin*-containing viruses) or *Xho*I (for *bcl-2*-containing viruses), transcribed in vitro with SP6 DNA-dependent RNA polymerase, and transfected into BHK cells by using Lipofectin according to the manufacture's instructions. Twenty-four hours after transfection, virus particle-containing supernatants of transfected cell monolayers were collected, frozen in aliquots at 70°C. and used for all subsequent experiments. Titers of stock viruses were determined by plaque assay titration on BHK-21 cells.

Animal Experiments:

Ten-day-old litters of CD1 mice were inoculated intra-cerebrally into the right cerebral hemisphere with 1,000 PFU of each recombinant virus in 0.03 ml of Hanks' balanced salt solution. For mortality experiments, three to six separate litters were inoculated with each virus, and mortality was determined by daily observation of the mice for 3 weeks after infection. For virus titration and histopathology experiments, three to six mice per experimental group were sacrificed at days 1, 2, and 4 after inoculation. The right cerebral hemisphere was dissected and stored at -70°C. and freeze-thawed tissues were used to prepare 10% homogenates in Hanks' balanced salt solution for plaque assay titration.

The left cerebral hemisphere was fixed by immersion in 3%
paraformaldehyde.

Histopathology:

5   Paraformaldehyde-fixed mouse brains were embedded in
paraffin, and a series of 4-μm parasagittal sections were cut
at the level of the olfactory bulb, extending caudally from
the bulb to the cerebellum and medulla. For each brain,
sequential sections were stained by hematoxylin and eosin to
10 detect histopathology, in situ end labeling (ISEL) to detect
apoptotic nuclei, in situ hybridization to detect Sindbis
virus RNA, and immunoperoxidase to detect flag-Beclin protein
expression. ISEL and in situ hybridization were performed
by methods identical to those described previously for
15 SIN/*bcl-2* infected mouse brains. Immunoperoxidase staining
to detect flag-Beclin protein expression in SIN/*beclin-*,
SIN/*beclin*ΔBcl-2BD-, and SIN/*beclin*stop-infected mouse brains
was performed by using the monoclonal anti-flag M2 (5 μg/ml;
VWR) and the avidin-biotin peroxidase method (Vectastain ABC
20 kit; Vector Laboratories) according to the manufacture's
instructions.

The number of virus RNA-positive and ISEL-positive cells in
each brain section was quantitated with Image-ProPlus
25 software. To calculate the number of positive cells per
brain section, nonoverlapping 0.25-mm² microscopic fields
spanning the entire brain section were scanned with a 10X
objective and constant settings for brightness, contrast, and
threshold values for positive events. The number of positive
30 events (i.e., RNA-positive cells or ISEL-positive cells) for
each brain section was determined by adding the sum of all
individual fields analyzed. The total number of positive
events per brain section was divided by the total area of the
brain section to yield the average number of RNA-positive or
35 ISEL-positive cells per square millimeter of brain.

Sections of hippocampus and anterior cortex from an adult
human were stained with 843, a polyclonal antibody against
a human Beclin peptide corresponding to aa 1 to 15 (1:200
dilution: Eurogenetics, Seraing, Belgium), and human Beclin
5   was detected by the avidin-biotin peroxidase method.

Plasmid Transfection:

Plasmids pSG5/*bcl-2* and pSG5/flag-*beclin* or pSG5/*bcl-2* and
pSG5/flag-*beclin*ΔBcl-2BD (1 μg of each) were transiently
10 transfected into COS7 cells by using Superfect (Qiagen)
according to the manufacture's instructions.

Protein Detection:

For immunofluorescence studies, COS7 cells were fixed 24 h
15 after transfection 100% ethanol. Expression of flag-Beclin
and flag-mutant BeclinΔBcl-2BD mutant constructs was detected
with an anti-flag epitope antibody (M2:1:20: VWR) and
fluorescein isothiocyanate (FITC)-conjugated horse anti-mouse
immunoglobulin G. Bcl-2 expression was detected with a
20 polyclonal rabbit anti-Bcl-2 antibody (1:100; Pharmingen) and
rhodamine-conjugated goat anti-rabbit antibody. SERCA
(endoplasmic reticulum Ca^{2+} ATPase) was detected with an
anti-SERCA antibody (1:500: Research Design, Inc.) Western
blot analysis to detect flag-Beclin expression in BHK cells
25 infected with SIN/*beclin*, SIN/*beclin*stop, and SIN/*beclin*ΔBcl-
2BD was performed with either antibody M2 (20 μg/ml) or anti-
human Beclin peptide antibody 843 (1:200) and enhanced
chemiluminescence detection as instructed by the manufacturer
(Amersham).

30

FRET:

Fluorescence resonance energy transfer (FRET) microscopy was
performed as previously described on COS7 cells cotransfected
with *bcl-2* and *beclin* expression vectors. The donor (FITC)
35 filter set had the following parameters: excitation (ex) =

450 to 490 nm; dichroic mirror (dm) = 510 nm; emission (em)
= 590 long pass. The acceptor (rhodamine) filter set had the
following parameters: ex = 546 nm; dm = 580 nm; em = 590 long
pass. Images obtained with these two filter sets were used
to directly quantify the intensities of each fluorophore.
The signal recorded from the FRET filter set (ex = 450 to 490
nm; dm = 580 nm; em = 590 nm long pass) is from energy that
has transferred from FITC to rhodamine molecules. A mapping
program described previously was used to map fluorescent
cells and to quantify the intensity within each cell.
Quantitative analysis of these mapped images required solving
three equations, one for each filter set, which accounted for
the excitation and detection of both labels in all three
filter sets as well as the concentrations of the donor and
acceptor molecules and the probability of transfer. The
measured quantities are expressed as follows, in which the
first letter (uppercase) indicates the filter set (A.
acceptor; F. FRET; D. Donor) and the second letter
(lowercase) indicates the labels present (a. acceptor alone;
f. acceptor and donor; d. donor alone). A solution of the
equation is $E = 1/[aconc(RK + 1)]$, where $aconc = (AdFf -
FdAf)/[(AdFa/Aa) - Fd]$; $R = (DaFf/Fa - Df)/[aconc ((Fa/Aa) -
FdDa/DdAa) - F = FdDf/Dd]$; and K is proportional to the
product of the ratio of the quantum yield of the two labels
and the ratio of the absolute detection efficiencies of the
two labels.

Nucleotide Sequence Accession Numbers:

The GenBank accession numbers for human and mouse *beclin* are
AF077301 and AF077302, respectively.

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FOURTH SERIES OF EXPERIMENTS:

Figure 22 shows the growth curve of the virus, a
gammaherpesvirus 68, in human MCF7 breast carcinoma cells
5 that are transfected with an empty vector (MCF7/control
cells) or with a vector expressing human *beclin* (MCF/*beclin*
cells). The results show that there is an approximately 10-
fold decrease in the amount of virus in cells which express
beclin as compared to the control cells. These data indicate
10 that *beclin* may also have antiviral effects against members
of the herpesvirus family.

In other studies, we have shown that there is a direct
interaction between Beclin and Bcl-2-like proteins that are
15 encoded by gammaherpesvirus 68, Kapsosi's Sarcoma Herpesvirus
(HHV8) and Epstein Barr virus.

FIFTH SERIES OF EXPERIMENTS

Genetic pathways regulating cellular homeostasis may be
important determinants of viral pathogenesis. We have shown
5 that overexpression of several anti-apoptotic genes in
virally-infected neurons protects suckling mice from fatal
alphavirus encephalitis, suggesting an important role of
virus-induced neuronal apoptosis in disease pathogenesis.
To further understand the molecular pathways governing
10 neuronal apoptotic death, we performed a yeast two hybrid
screen of a mouse brain library, using the anti-apoptosis
gene, bcl-2 as a bait. We identified and characterized a
novel bcl-2 interacting gene, termed beclin. Beclin plays
a role in bcl-2-mediated protection against alphavirus-
15 induced apoptosis in vitro, and beclin overexpression in vivo
protects mice against fatal alphavirus encephalitis. Beclin
is a candidate tumor suppressor gene which inhibits cellular
proliferation in vitro and tumorigenesis in vivo, perhaps by
acting as a negative regulator of G2/M. Overexpression of
20 a dominant negative mutant of cdc2, a cyclin-dependent kinase
required for the G2/M transition, also protects mice against
fatal alphavirus encephalitis. These observations suggest
that cell cycle regulators, particularly those involved in
G2/M, may be important for virus-induced neuronal death in
25 vivo.

SIXTH SERIES OF EXPERIMENTS

The process of autophagy, or bulk degradation of cellular
proteins through an autophagosomic-lysosomal pathway, is
important in normal growth control and may be defective in
5   tumor cells.   However, little is known about the genetic
mediators of autophagy in mammalian cells and whether genetic
mediators   of   autophagy   influence   tumor   development.
Recently, 14 genes, the *apg* genes, have been identified in
S. cerevisiae that are required for yeast autophagy[1].   The
10   mammalian gene encoding Beclin 1[2], a novel Bcl-2-interacting,
coiled-coil protein, shares structural similarity with the
yeast autophagy gene product, Apg6/Vps30p (3,4), and is
monoallelically deleted in 40-75% of sporadic human breast
cancers and ovarian cancers [5-11].   Here we show, using gene
15   transfer techniques, that *beclin 1* promotes autophagy both
in autophagy-defective yeast with a targeted disruption of
apg6/vps30 and in autophagy-defective human MCF7 breast
carcinoma cells. The autophagy-promoting activity of *beclin*
1 in MCF7 cells is associated with an ability to inhibit MCF7
20   cellular   proliferation,   *in   vitro*   clonigenicity,   and
tumorigenesis in nude mice.   Furthermore, endogenous Beclin
1 protein expression is frequently undetectable or low in
malignant human breast epithelial cell lines and tissue, but
is expressed ubiquitously at high levels in normal breast
25   epithelia.   Thus, *beclin 1* is a mammalian autophagy gene with
tumor suppressor function that is expressed at decreased
levels in human breast cancer.   These findings indicate that
there is an evolutionarily conserved genetic link between
autophagy and tumor suppressor pathways, and raise the
30   possibility that decreased expression of autophagy proteins
may contribute to the development or progression of breast
and other human malignancies.

Autophagy is an evolutionarily conserved process that occurs
in both yeast and mammalian cells in which there is bulk
degradation of cellular contents via an
autophagosomal-lysosomal pathway (reviewed in [12]). The
5 process of autophagy liberates free amino acids and
nucleotides and enable cells to survive under nutrient
deprivation conditions as well as to undergo structural
remodeling during differentiation. Protein degradation
through an autophagy pathway has also been postulated to
10 serve as a mechanism for negative regulation of cell growth.
In support of this theory, cancerous transformation *in vitro*
is associated with decreased rates of autophagic
degradation[13,14]. Malignant cell lines display less autophagic
activity than their normal counterparts and are less prone
15 to increase autophagic activity in response to serum
deprivation or high cell density[15-18]. Furthermore, in rat
liver carcinogenesis models, there is an inverse correlation
between stage of malignancy and autophagic activity[19].
However, it is not known whether reduced autophagy in tumor
20 cells directly contributes to deregulated cell growth, or
alternatively, is the result of malignant transformation.

Genetic screens have identified 14 genes that are required
for autophagy in yeast, but no mammalian autophagy genes have
25 as-of-yet been identified[1,3,20-22]. Human Beclin 1 was recently
isolated using a yeast two-hybrid screen with Bcl-2 as a
bait[2], and shares 24.4% amino acid identity (and 39.1%
conservation) with the yeast autophagy gene product,
Apg6/Vps30p. Yeast disrupted of *apg6/vps30* are defective
30 both in their ability to undergo nitrogen deprivation-induced
autophagy[3] as well in their ability to properly sort
selective vacuolar proteins[4]. To determine whether Beclin 1
is a functional homolog of Apg6/Vps30p, we investigated
whether *beclin 1* expression could restore autophagic activity
35 in *apg6/vps30*-disrupted yeast. *Apg6/vps30*-disrupted yeast
(strain JCY300)[4] were first transformed with the yeast
expression vector, pRS424.*vps30* which contains the complete

apg6/vps30 gene. The open reading frame of *apg6/vps30* was then replaced by homologous recombination with the open reading frame of human *beclin 1* in the JCY3000 + pRS424.*vps30* transformants to generate yeast clones (referred to as

5   JCY3000 + pRS424.*beclin 1*) that express human flag epitope-tagged Beclin 1 (Fig. 23a) under the control of *apg6/vps30* regulatory regions. Four hours following transfer to nitrogen-starvation media, we compared the percentage of cells with autophagic bodies within the yeast vacuole in

10   isogenic wild-type yeast (strain SEY620)[4], *apg6/vps30*-disrupted yeast (strain JCY3000)[4], *apg6/vps30*-disrupted yeast transformed with pRS424.*vps30*, *apg6/vps30*-disrupted yeast transformed with pRS424.*beclin 1*, and *apg6/vps30*-disrupted yeast transformed with an empty

15   pRS424 vector. In the presence of PMSF (a protease inhibitor that facilitates detection of autophagic bodies by blocking their degradation[21]), numerous cells with autophagic bodies within the vacuole were seen in wild-type yeast, and *apg6/vsp30* and *beclin 1*-transformed Δ*apg6/vps30* yeast (Fig.

20   23b, 23c). In contrast, significantly fewer cells were seen with autophagic bodies within the vacuole among non-transformed Δ*apg6/vps30* yeast or Δ*apg6/vps30* yeast transformed with empty pRS424 (p<0.0001, analysis of variance). Thus, *beclin 1* complements *apg6/vps30* and

25   restores autophagy in autophagy-defective Δ*apg6/vps30* yeast.

The *beclin 1* gene maps to a tumor susceptibility locus on human chromosome 17q21[23,24] that is monoallelically deleted in up to 40-75% of cases of sporadic ovarian and breast

30   carcinomas [5-11,25]. This observation, coupled with the homology between *beclin 1* and *apg6/Vps30*, led us to hypothesize that *beclin 1* is a mammalian autophagy gene with tumor suppressor function. To evaluate this hypothesis, we investigated the effects of *beclin 1* gene transfer on the

35   autophagic activity and growth properties of MCF7 human

breast carcinoma cells. MCF7 cells were originally derived
from a patient with 17q21 loss-of-heterozygosity [26] and do
not express detectable levels of endogenous Beclin 1 (Fig.
25a). To permit conditional expression of a potential
5   anti-proliferative gene, we stably transfected MCF7 cells
with a tetracycline-repressible vector, pTC[27], containing
flag-epitope tagged human Beclin 1 (referred to as
MCF7.*beclin1* clones) or no insert (referred to as
MCF7.control clones). Seven MCF7.*beclin1* clones which
10   expressed flag-Beclin 1 following tetracycline withdrawal
(Fig.3a) were chosen for further phenotypic analysis.

Electron microscopic analysis was performed of two
MCF7.control clones and two MCF7.*beclin1* clones which were
15   either maintained in normal growth conditions or subjected
to serum and amino acid deprivation for three and a half
hours (Fig.24). In normal cultured cells, serum and amino
acid deprivation is a potent inducer of autophagy (reviewed
in [28]), which can be recognized at the ultrastructural level
20   as double membrane vacuolar structures containing visible
cytoplasmic contents[29-31]. To quantitate the amount of
autophagy in different MCF7 clones, we examined 100 cells of
each clone and determined the mean number of autophagic
vacuoles per cell (Fig. 24f). We found that basal levels of
25   autophagy were significantly higher in MCF7.*beclin1* as
compared to MCF7.control clones ($p < 0.0001$, analysis of
variance; see Fig. 24a, 24b, 24f). Furthermore, the mean
number of vacuoles per cell did not increase following serum
and amino acid deprivation in MCF7.control cells (Fig 24a,
30   24c, 24f), whereas serum and amino acid deprivation induced
a significant increase in autophagy in MCF7.*beclin1* clones
($p < 0.0001$, t-test; Fig 24b, 24d, 24f). This increase was
inhibited by pre-treatment with 3-methyladenine, a nucleotide
derivative that inhibits the earliest stages of autophagosome
35   formation[32]. These results indicate that MCF7 human breast
carcinoma cells that lack Beclin 1 expression are defective
in their capacity to undergo autophagy in response to

nutrient deprivation. Enforced *beclin 1* expression increases
the bassal levels of autophagy in MCF7 cells as well as
restores their ability to undergo nutrient
deprivation-induced autophagy.

5   To evaluate the effect of *beclin 1* on MCF7 growth properties,
we compared morphology, cellular proliferation rates,
clonigenicity *in vitro*, and tumorigenicity *in vivo* of
MCF7.*beclin1* and MCF7.control clones. MCF7.*beclin1* clones
10   displayed several morphologic characteristics consistent with
a less malignant phenotype, including flatter appearance,
larger size, firmer attachment to tissue culture plate, and
increased contact inhibition (see electron and light
photomicrographs of representative clones, Fig. 24a-b, Fig.
15   25b). As would be predicted based upon the
autophagy-promoting effects of *beclin 1*, all MCF7.*beclin1*
clones proliferated at a slower rate than MCF7.control clones
(Fig. 25c). (The lower rate of proliferation of MCF7.*beclin1*
cells could not be explained by an increase in cell death,
20   since cell viability determined by trypan blue staining at
identical time points was similar among MCF7.*beclin1* and
MCF7.control cells [data not shown]. All MCF.*beclin1* clones
were also severely impaired in their clonigenicity *in vitro*,
as compared to MCF7.control clones which formed colonies in
25   soft agar with high efficiency (p<0.0001, Chi-Square) (Fig.
3d). Moreover, the incidence of tumor formation of two
MCF7.*beclin1* clones that were injected into nude mice was
significantly lower than that of three MCF7.control clones
(14-25% vs. 75-89%, p<0.0001; Chi-square) (Fig. 25e). The
30   few tumors that did develop in mice injected with
MCF7.*beclin1* clones had very few flag-Beclin 1 immunoreactive
cells whereas almost all MCF7.*beclin1* cells were
immunoreactive prior to injection into nude mice, indicating
that tumor formation was associated with loss of Beclin 1
35   protein expression (see representative example, Fig. 25f).
Taken together, these data demonstrate that Beclin 1 can

function as a negative regulator of mammary cell growth and
tumorigenesis.

The frequent allelic deletions of *beclin 1* in human breast
cancer, coupled with its autophagy-promoting and tumor
suppressor effects, raise the possibility that functional
inactivation of *beclin 1* may be important in breast cancer
development or progression. A previous study did not
identify any coding region mutations in 10 human breast
carcinoma cell lines (including 8 cell lines with net allelic
deletions of *beclin 1* identified by FISH analysis) and did
not identify any abnormalities in *beclin 1* mRNA expression
in human breast carcinoma cell lines [33]. Although these
findings do not exclude genomic DNA mutations of *beclin 1* in
human breast cancer, they do suggest that such mutations may
not be frequent. However, certain tumor suppressor genes do
not demonstrate bi-allelic mutations in cancer (and fulfill
the Knudson two-hit criteria[34] for classification as a tumor
suppressor gene) but still demonstrate haplo-insufficiency
for tumor suppression. The prototypic example, p27 [35], is
mono-allelically deleted [36-38] and has low protein expression
[39-42] in a high percentage of human cancers; the low protein
expression is thought to result from decreased protein
stability [40] rather than genetic alterations or altered
transcription.

To evaluate whether down-regulation of Beclin 1 protein
expression is a mechanism of functional inactivation in human
breast cancer, we performed Western blot analysis of human
breast carcinoma-derived cell lines and matched normal and
malignant breast tissue from patients with invasive sporadic
breast carcinoma. Of 11 human breast carcinoma cell lines
examined, only three demonstrated detectable Beclin 1 protein
expression (see representative samples in Fig. 26a). Of 17
pairs of equivalent volumes of matched normal and malignant
breast tissue, 15 samples had higher levels of Beclin 1
expression in normal as compared to tumor tissue, whereas 16

pairs had higher levels of expression of control proteins
(e.g. actin, cytokeratin) in the tumor tissue (see
representative samples in Fig. 26b). These latter findings
demonstrate the despite higher overall levels of protein in
5   breast tumors (which consist primarily of malignant
epithelial cells) as compared to normal breast tissue (which
consists primarily of .fat cells), specific expression of the
Beclin 1 protein is usually decreased.

10   To confirm that the decreased levels of Beclin 1 protein
expression in tumors reflected a loss of expression in
epithelial cells, we performed immunohistochemical staining
of paraffin-embedded sections from 10 of the matched normal
and malignant breast samples analyzed by Western blot. We
15   found that in all 10 cases, there was strong Beclin 1
immunoreactivity in all normal breast epithelial cells (see
examples in Fig.26c) (as well as in the media layer of smooth
muscle). However, in five of the ten cases, Beclin 1
immunoreactivity was absent in all of the malignant
20   epithelial cells. Immunoperoxidase staining of an additional
22 of cases of breast carcinoma revealed decreased expression
of Beclin 1 in malignant as compared to normal breast
epithelial cells in 13 cases. Thus, *beclin 1* is expressed
in normal breast epithelial cells, but its expression is
25   commonly decreased in malignant breast epithelial cells.

Reduced levels of Beclin 1, an autophagy and tumor suppressor
protein, in human breast carcinoma suggests that therapeutic
compounds that increase Beclin 1 protein expression in human
30   breast carcinoma cells may be effective treatments for human
breast cancer. This hypothesis was evaluated by determining
whether tamoxifen, an anti-estrogen compound that is used in
treating breast cancer, increases the expression of Beclin
1 in MCF7 human breast carcinoma cells. The levels of Beclin
35   1 antibody, 843, at serial time points after treatment with
10mm of tamoxifen. The results (Figure 27) indicate that
Beclin 1 protein expression is barely detectable in untreated
MCF7 cells and that within 72 hours after tamoxifen

treatment, Beclin 1 protein expression is significantly
increased in MCF7 cells. The ability of tamoxifen, an
effective anti-breast tumor agent, to increase Beclin 1
expression in MCF7 human breast carcinoma cells supports the
5 hypothesis that induction of Beclin 1 expression in human
breast cancer may be therapeutically effective.

In summary, data indicates that *beclin 1* is the first
identified mammalian gene with a role in mediating autophagy.
10 Prior studies have indicated that signalling through the S6
kinase pathway exerts inhibitory effects on mammalian
autophagy [43], but no genes have been identified that play a
role in the execution of autophagy in mammalian cells. Human
homologs exist of other yeast autophagy genes [44,45] and given
15 the functional evolutionary conservation between *beclin 1* and
apg6/vps30, it seems likely that these human homologs will
also be found to play a role in mammalian autophagy.
Modification of one of the human homologues, human Apg5p, in
apoptotic cells[44], has led others to postulate that yeast
20 autophagy and mammalian apoptosis may share common pathways
[46]. Interestingly, Beclin 1 interacts with the apoptosis
regulator, Bcl-2 [2], further suggesting a possible
interrelationship between autophagy and apoptosis. However,
at least in the context of a CNS viral infection, *beclin 1*
25 has anti-apoptotic, not pro-apoptotic effects [2], consistent
with a model in which autophagy enables stressed cells to
survive rather than a model in which autophagy machinery is
used to execute apoptotic death.
A common feature of malignant cells is that they fail to
30 respond to normal growth inhibitory and autophagy-stimulatory
signals such as nutrient deprivation and high density [15-18].
Enforced expression of an autophagy gene not only restores
nutrient deprivation-induced autophagy in breast carcinoma
cells but also suppresses their malignant phenotype suggest
35 that autophagy is a fundamental mechanism for preventing the
deregulated growth of tumor cells. Furthermore, the frequent
monoallelic deletions and decreased expression of *beclin 1*

in human breast cancer suggest that specific molecular
alterations in autophagy pathways may contribute to
tumorigenesis. It may therefore be possible to develop new
anti-cancer therapeutic strategies that increase the
expression of autophagy genes such as *beclin 1*, and thereby,
restore normal growth control.

MATERIALS AND METHODS

Yeast strains, media, and genetic methods. The *Saccharomyces cerevisiae* strains used for cloning, immunochemical analysis,

5   and autophagy assays were SEY6210 (MATαleu2-3, 112 ura3-52 hisΔ200 trp1-Δ901 lys2-801 suc2-Δ9) and JCY3000 (SEY6210 Δvps30::HIS3). JCY3000 yeast were transformed with pRS.vps30 with the procedure of Eble et al [47] and flag-*beclin 1* was substituted for the open reading frame of *apg6/vps30* in

10   pRS.vps30 using recombination-mediated PCR-directed plasmid construction *in vivo* [48]. Yeast were grown in either YEPD, in synthetic complete medium, SD, lacking trp for transformation selection, or in SD(-N) for starvation experiments, consisting of 2% glucose with 1.7g/l of yeast nitrogen base

15   without amino acids and without ammonium sulfate.

Mammalian cell lines and transfections. Human breast carcinoma cell lines were obtained from ATCC and maintained according to ATCC instructions. MCF7 cells were transfected

20   using liposome-mediated transfer with the plasmid, pTC[27], containing flag epitope-tagged human *beclin 1* cloned into the Nhe I site or no insert. Stable transfectants were selected for with 300 μg/ml hygromycin and maintained in 2 μg/ml tetracycline; tetracycline was withdrawn five days before all

25   experiments.

Immunochemical procedures. Yeast and mammalian cell lysates were subjected to SDS-PAGE, followed by immunoblotting with anti-flag monoclonal (M2, VWR; 20 μg/ml) or polyclonal

30   antibody (Zymed, 1:200 dilution), polyclonal anti-Beclin 1 antibody 843[2] (1:200 dilution), or anti-actin (Boehringer Mannheim, 1:400 dilution). Immunoperoxidase staining to detect flag-Beclin 1 and endogenous Beclin 1 expression was performed using M2 (5 μg/ml) or 843 (1:100), respectively,

with the ABC method according to the manufacturer's instructions (Vector Laboratories).

Autophagy analysis. Yeast strains were grown overnight in
5 YEPD or liquid SD-trp, transferred to SD (-N) at a concentration of 2×10^7 cells/ml, incubated for four hours in the presence or absence of 1mM PMSF, visualized using DIC optics with a Plan-Apochromat 100x 1.4 NA objective, and imaged with a cooled CCD camera using IP Lab software. MCF7
10 cells were grown in normal media or serum and amino-acid free media, fixed with 2.5% glutaraldehyde, postfixed with 1% OsO_4, and embedded in Lx-112 (Ladd Research Industries, Inc.) and Embed-812 (E.M.S.). Thin sections were cut on MT-700 RMC, stained with uranyl acetate and lead citrate, and
15 examined by transmission electron microscopy using a JEOL JEM-1200 EXII.

Proliferation, clonigenicity and tumorigenicity assays. MCF7 clones were seeded in 96 well plates at a density of 5×10^3
20 cells per well, and cell proliferation was measured at serial time points using the MTT Cell Proliferation Kit (Boehringer Mannheim) according to the manufacturer's instructions. MCF7 clones were plated in triplicate at a density of 5×10^4 cells per 35 mm-well in semi-solid medium (soft agar) as
25 described [49] and colonies were counted at 21 days. Five week-old NCR nude mice (Taconic Farms) were implanted with slow release estrogen pellets (1.7 mg/60 day, Innovative Research of America) and injected subcutaneously with 5×10^6 tumor cells. Animals were necropsied eight weeks after
30 injection for gross and histologic confirmation of tumor and analysis of flag Beclin 1 protein expression.
Autophagy is an evolutionarily conserved process that occurs in both yeast and mammalian cells in which there is bulk degradation of cellular contents via an
35 autophagsomal-lysosomal pathway. The process of autophagy liberates free amino acids and nucleotides and enables cells to survive under nutrient deprivation conditions as well as

to undergo structural remodeling during differentiation. In
mammalian cells, the process of autophagy is thought to occur
in distinct stages, involving invagination of the rough
endoplasmic reticulum to form an early autophagosome; fusion
5   of the autophagosome with a vesicle containing lysosomal
autophagosomethe formation membrane proteins (e.g. LGP120 and
H⁺-ATPase)   to   become   an   acidic   late   autophagosome;
acquisition of acid hydrolases and hydrolysis of its vacuolar
contents; and   transformation into a residual body.   In
10 yeast, the sequence of events is essentially the same, with
formation of an autophagosome; fusion of the autophagosome
to the vacuole, which is equivalent to a lysosome in
mammalian cells; delivery of the inner membrane structures
of the autophagosome to the vacuole as autophagic bodies; and
15 destruction of the autophagic bodies by yeast proteinases.

For decades, it has been postulated that protein degradation
through an autophagy pathway may serve as an important
mechanism for negative regulation of cell growth. Support
20 for this hypothesis stems from the following observations:
In addition to changes in rates of protein synthesis, cell
growth rate can be modulated by changes in the rate of
protein degradation.   Negative control of cell growth
through protein degradation is generally exerted by an
25 autophagic pathway.   Cancerous transformation in vitro may
be associated with changes in protein degradation. Malignant
cell lines usually display lower protein degrading activity
than their normal counterparts and may be less prone to
increase their degradation rate in response to serum
30 deprivation or high cell density.   In   rat   liver
carcinogenesis models, a mild reduction in lysosomal protein
degradation and autophagic activity is observed at a
preneoplastic stage and more significant reduction is
observed in primary hepatocellular tumor cells .

35

Together, these observations raise the interesting possibility that defects in autophagy pathways may contribute to deregulated cell growth. However, all data to date regarding the relationship between autophagy and negative

5 growth control has been of a correlative nature; i.e. decreased autophagy has been observed in cells with increased growth rates but it is not known whether the reduced autophagy in tumor cells is a cause or effect (or associated epiphenomenon) of malignant transformation. Further studies

10 are required to evaluate whether there is a causal relationship between autophagy dysregulation and tumorigenesis.

The recent characterization of autophagy in yeast has

15 provided the opportunity to use genetic approaches to investigate the molecular control of autophagy. Genetic screens in the yeast *Saccharomyces cerevisiae* identified 14 genes, termed *apg* genes, that are required for yeast autophagy. Mutation or disruption of these genes prevents

20 yeast from forming autophagic bodies when subjected to nutrient deprivation, from surviving during conditions of nutrient deprivation, and from undergoing sporulation/differentiation. The original cloning of the *apg* genes revealed little about their function. However, recent

25 advances have shed more light on the possible functions of the individual *apg* genes. First, Matsuura et al. have shown that Apg1p is a novel type of serine/threonine protein kinase, providing evidence for involvement of protein phosphorylation in the regulation of autophagy. Second,

30 Mizushima et al. have shown that a unique covalent-modification system is essential for autophagy to occur in yeast. The carboxy-terminal glycine residue of Apg12p is conjugated to a lysine residue of Apg5p, and Apg7p is a ubiquitin-E1 like enzyme that is required for the

35 conjugation of Apg12p and Apg5p. Third, the yeast autophagy

gene product, Apg6p, was independently identified as Vps30p
in a screen for yeast proteins involved in vacuolar protein
sorting. Yeast with a targeted disruption of apg6/vps30 are
unable to properly sort selective vacuolar hydrolases, such
5 as carboxypeptidase Y.

Although autophagy has been recognized as an important
process in mammalian cells for the past four decades,
virtually nothing is known about the genes involved in
10 mammalian autophagy. The existence of human homologues of
some of the recently cloned yeast autophagy genes provides
new opportunities to make important discoveries about the
molecular mediators of mammalian autophagy. The apg5 and
apg12 genes both have human homologues, suggesting that there
15 is a conserved pathway of ubiquitin-dependent protein
degradation in yeast and mammalian cells. In addition, our
laboratory has recently cloned and characterized the human
homolog of the yeast autophagy gene, apg6/vps30, which is a
novel gene termed beclin 1. We isolated beclin 1 by virtue
20 of the interaction of its encoded gene product with the
anti-apoptotic protein, Bcl-2, in a yeast two-hybrid screen.
In previously published studies, we demonstrated that beclin
1 is ubiquitously expressed in mouse and human tissues and
that overexpression of wild-type Beclin 1, but not Beclin 1
25 lacking the Bcl-2 binding domain, inhibits Sindbis
virus-induced apoptosis in neurons. In addition to cellular
Bcl-2 family members, we have also found that viral
Bcl-2-like proteins encoded by oncogenic gammaherpesviruses
interact with Beclin 1.
30

Yeast and mammalian autophagy is regulated by a variety of
environmental   factors,   the   most   influential   being
availability of amino acids.   The formation of the
autophagosome is modulated by a group of regulatory amino
35 acids: leucine, glutmaine, proline, methionine, histidine,

tryptophan, tyrosine and phenylanalanine. When these amino
acids are removed from the culture medium, both the rate of
protein and RNA degradation and the number of autophagic
vacuoles increase. Amino acids are considered to be primary
5   regulators of autophagy, since they can override most forms
of induced proteolysis in the absence of hormones. However,
hormones and growth factors also play a role in the
regulation of autophagy. For example, insulin inhibits the
degradation of proteins and RNA and the formation of
10 autophagosomes in many cell types. Glucagon, isoproterenol
and cyclic AMP stimulate protein degradation and autophagy
in liver cells, but have opposite effects in cardiac
myocytes. In addition, proteolysis induced by nutrient
stress can be suppressed by EGF and IGF-1 in many cell types

15

Recent evidence suggests that these environmental/hormonal
factors may regulate autophagy through an evolutionarily
conserved signalling pathway involving TOR in yeast and RAFT1
in mammalian cells. A model is emerging in which TOR, a
20 phosphatidylinositol kinase homologue and its mammalian
counterpart, RAFT1, are thought to negatively regulate
induction of autophagy by activation of p70 S6
serine/threonine kinase which in turn, phosphorylates the
ribosomal protein S6. Phosphorylation of ribosomal protein
25 S6 is simultaneously inhibitory for autophagy and stimulatory
for protein synthesis and cell cycle progression through G1
in response to mitogenic signals. Consistent with this
model, treatment of cells with autophagy inhibitory stimuli
such as insulin and amino acids is associated with linear
30 increases in S6 phosphorylation, and linear decreases are
seen in p70 S6 kinase activation and S6 phosphorylation under
autophagy promoting conditions. The evidence for a negative
regulatory role of TOR in yeast autophagy is two-fold.
First, rapamycin, an inhibitor of TOR function, induces
35 autophagy even in yeast cells growing in nutrient-rich

medium. Second, a temperature sensitive TOR mutant leads to
induction of autophagy at a nonpermissive temperature. (Of
note, rapamycin inactivation of TOR in yeast also induces a
G0 growth arrest. The site of action of TOR is upstream of
5   the Apg proteins, as rapamycin does not induce autophagy in
yeast with disrupted apg genes.

These observations suggest that a TOR/RAFT1-S6 kinase
signalling pathway may simultaneously regulate the balance
10 between cell proliferation in response to mitogens and
negative cell growth via autophagy. The TOR/RAFT1 signal
transduction pathway is thought to provide an important
checkpoint control, linking amino acid sufficiency to the
control of peptide chain initiation. Abnormal activation of
15 S6 kinase activity is postulated to be a potential mechanism
for uncontrolled cell growth and oncogenesis.   An
interesting possibility is that inactivation of downstream
targets of TOR involved in the autophagy arm of the pathway,
such as the Apg proteins, could also contribute to
20 deregulated cell growth.
In addition to a role in apoptosis regulation , beclin 1, the
human homolog of the yeast autophagy gene, apg6/vps30, is a
candidate tumor suppressor gene.   We originally became
interested in the possibility that beclin 1 is a candidate
25 tumor suppressor gene because it maps to a region of
chromosome 17q21 that is frequently monoallelically deleted
in sporadic human breast and ovarian cancer. While the tumor
suppressor gene BRCA1 maps to this region, BRCA1 is thought
to only be important in the familial and not sporadic forms
30 of these diseases . Most cancer geneticists believe that one
or more tumor suppressor genes within this region are
important in the sporadic forms of breast and ovarian cancer.

Preliminary data obtained to date raise the possibility that
35 beclin 1 may be a tumor suppressor gene that demonstrates
haplo-insufficiency. (i.e. monallelic deletion confers

susceptibility   to   tumorigenesis,   a   concept   recently
demonstrated with p27 +/- mice)   Several previous studies
have shown deletions of the *beclin 1-* containing region of
chromosome 17q21 in up to 50% of cases of sporadic breast
cancer   and   75%   of   cases   of   sporadic   ovarian   cancer
((including some cases that did not have deletions of the
BRCA1 region of chromosome 17q21]).   In human breast cancer
cell lines, we confirmed by FISH analysis that *beclin 1* is
frequently allelically deleted; 9 of 22 cell lines examined
contained net *beclin 1* deletions.   In a mutation analysis of
8 cases of sporadic ovarian cancer with 17q21 LOH   and 10
breast cancer cell lines with net *beclin 1* deletions , we
have not detected any mutations within the *beclin 1* coding
region.   However, we have found that Beclin 1 protein is
expressed ubiquitously in normal breast epithelial cells, but
that its expression is frequently absent or decreased in
human breast cancer cell lines and in human breast cancer
tissue.   Furthermore, in the preliminary data section of
this proposal, we present evidence that enforced Beclin 1
expression in human MCF7 breast carcinoma cells delays cell
proliferation, interferes with anchorage-independent growth,
and decreases tumor formation in nude mice. Thus, *beclin 1*
is frequently deleted in human breast cancers, its expression
is frequently down-regulated in human breast cancers, and it
can suppress mammary tumorigenesis.   Taken together, these
observations suggest that *beclin 1* is a strong candidate
tumor suppressor gene.   (In the absence of evidence of
bi-allelic   inactivation   of   beclin 1   in   human   cancers,
functional studies in beclin 1 -/+ mice will be required for
more definitive proof that it is a haplo-insufficient tumor
suppressor.

Impairment of autophagy may contribute to the development or
progression of human tumors.   However, there has been no
direct evidence implying a role for deregulation of autophagy
in tumorigenesis; the reductions in autophagy observed in

malignant cells could be secondary or epiphenomenon that are
a consequence of other changes in the cells. Our finding
that a mammalian homolog of a yeast gene required for
autophagy is a candidate tumor suppressor provides the first
5   suggestion of a possible genetic link between autophagic and
tumor suppressor pathways. In the preliminary data section,
we present evidence that *beclin 1* possesses an evolutionarily
conserved role in autophagy, that *beclin 1* has tumor
suppressor function in breast carcinoma cells, and that
10   *beclin 1* expression is down-regulated in breast cancer.
These data raise the possibility that the tumor suppressor
function of *beclin 1* may be related to a mechanism involving
autophagy induction and that down-regulation of autophagy
proteins may contribute to the development and/or progression
15   of breast and other human cancers. In this proposal, we plan
to further explore the relationship between the role of
beclin 1 in autophagy and tumorigenesis. Such studies are
expected   to   provide   fundamental   insights   about   the
interrelationship between autophagy and the mechanisms
20   underlying the growth of human tumors.

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What is claimed is:

1.   A   method   for   treating   cancer   which   comprises
      administering   to   the   subject   a   therapeutically
      effective amount of beclin so as to restore cell
      growth control.

2.   A method for treating cancer comprising inducing
      increased expression of beclin, thereby restoring
      cell growth control in the subject.

3.   A method for treating cancer comprising inducing
      increased expression of beclin, thereby restoring
      cell growth control in a cell.

4.   A   method   for   treating   cancer   which   comprising
      contacting a cell with an effective amount of beclin
      so as to restore cell growth control.

5.   A method for inhibiting viral replication comprising
      administering an effective amount of beclin with the
      virus infected cell, thereby inhibiting the viral
      replication.

6.   A method for inhibiting viral replication comprising
      contacting induction of the expression of Beclin with
      the virus infected cell, thereby inhibiting the viral
      replication.

7.   A method of treating a viral infection in a subject,
      wherein   the   viral   infection   is   alleviated   by
      administering a pharmaceutical composition to induce
      increased expression of beclin.

8.   The method of claim 5, wherein the beclin is full-
        length beclin.

9.   The method of claim 5, wherein the Beclin is a
        fragment of beclin.

10.   The method of claim 5, wherein the virus is an RNA
        virus.

11.   The method of claim 5, wherein the virus is a Sindbis
        virus.

12.   The method of claim 5, wherein the virus is a herpes
        virus.

13.   The method of claim 5, wherein the virus is influenza
        virus.

14.   The method of claim 5, wherein administering beclin
        comprises:
        (a) recovering infected cells from the subject;
        (b) introducing the isolated beclin into the cells; and
        (c) reintroducing the cells of step (b) into the subject
        so as to treat the infected subject.

15.   The method of claim 14, wherein the reintroduction
        comprises, topical, oral, aerosol, subcutaneous
        administration, infusion, intralesional, intramuscular,
        intraperitoneal, intratumoral, intratracheal,
        intravenous injection, liposome-mediated delivery or
        vector delivery.

16.   A method of diagnosing a predisposition to carcinoma in
        a subject comprising:
        a.   obtaining a nucleic acid sample from the subject;
                and

     b.   determining whether one or more of the subject's
beclin alleles or regulatory regions to those
alleles are deleted or different from the wild
type so as to reduce the subject's expression of
polypeptide controls cell growth.

17. The method of claim 16, wherein the control of cell growth is by suppression of tumor activity.

18. The method of claim 16, wherein the control of cell growth is by autophagy.

19. The method of claim 16, wherein the carcinoma is breast cancer.

20. The method of claim 16, wherein the carcinoma is ovary cancer.

21. The method of claim 16, wherein the carcinoma is prostrate cancer.

22. The method of claim 16, wherein one or more beclin alleles contain splice site junction mutations.

23. The method of claim 16, wherein there are one or more mutations in the 100 base pair region centered about the splice acceptor site consensus sequence located upstream of the exon spanning the nucleotides corresponding to postions 1042-1185 of figure 1B or one or more mutations in the 100 base pair region centered about the splice donor site consensus sequence located downstream of the exon spanning the nucleotides corresponding to positions 1042-1185 of figure 1B.

24. The method of claim 16, wherein the determining of step b comprises determining whether one or more beclin promoter regions differ from the wild type.

25.   The method of claim 16, wherein the determining of step
       (b) comprises determining whether one or more beclin
       promotor regions are hypermethylated.

26.   The method of claim 16, wherein the determining of step
       (b) comprises determining whether one or more beclin
       enhancer regions differ from the wild type.

27.   The method of claim 16, wherein the determining of step
       (b) comprises determining whether one or more beclin 5'
       or 3' nontranslated regions differ from the wild type.

28.   The method of claim 16, wherein the determining of step
       (b) comprises determining whether one or more beclin
       alleles have a premature termination codon.

29.   The method of claim 16, wherein the determining of step
       b comprises determining whether there is a premature
       termination codon in the nucleic acid corresponding to
       positions 1180-1240 of figure 1B.

30.   The method of claim 16, wherein the determining of step
       (b) comprises contacting the nucleic acid sample with
       a primer that specifically hybridizes to the junction
       between the exon corresponding to positions 1042-1185
       of figure 1B and the exon adjoining downstream thereto.

31.   The method of claim 16, wherein the determining of step
       b comprises contacting the nucleic acid sample with a
       primer that specifically hybridizes to the junction
       between the exon corresponding to positions 1042-1185
       of figure 1B and the exon adjoining upstream thereto.

32.   The method of claim 16, wherein the determining of step
       (b) comprises contactin the nucleic acid sample with a
       primer that specifically hybridizes to the junction
       between the exon corressponding to positions 1042-1185

of figure 1B and the intron adjoining downstream
thereto.

33. The method of claim 16, wherein the determining of step
     (b) comprises contacting the nucleic acid sample with
     a primer that specifically hybridizes to the junction
     between the exon corresponding to positions 1042-1185
     of figure 1B and the intron adjoining upstream thereto.

34. The method of claim 16, wherein a majority of the
     nucleotides corresponding to positions 1042-1185 of
     figure 1B have been deleted.

35. The method of claim 16, wherein the subject was
     preselected based upon an abnormality at chromosome
     position 171q21.

36. The method of claim 16, wherein the abnormality at
     chromosome position 17q21 comprises a deletion.

37. The method of claim 16, wherein the nucleic acid sample
     is obtained from the subject's blood.

38. A method of diagnosing a prediposiiton to carcinoma in
     a subject, comprising determinng whether the subject
     has an abnormal subcellular distribution of Beclin.

39. The method of claim 38, wherein the abnormal
     subcellular distribution of Beclin comprises Beclin
     being distributed diffusely in both the nucleus and
     cytoplasm.

40. The method of claim 38, wherein the carcinoma is breast
     cancer.

41. The method of claim 38, wherein the carcinoma is ovary
     cancer.

42. The method of claim 38, wherein th ecarcinoma is prostrate cancer.

43. A method of diagnosing a predispositoin to carcinoma in a subject, comprising contacting the contents of one or more of th esubject's cells with one or more antibodies or fragments there of specific to Beclin.

44. The method of claim 43, wherein one or more of the antibodies is the antibody with designation number 843 and one or more of the antibodies is the antibody with designation number 844.

45. The method of claim 43, wherein the carcinoma is breast cancer.

46. The method of calim 43, wherein the carcinoma is ovary cancer.

47. The method of claim 43, wherein the carcinoma is prostrate cancer.

48. The method of claim 43, wherein the antibody's failure to bind to Beclin indicates that the subject has a predisposition to carcinoma.

49. A pharmaceutical composition which comprises Beclin and a pharmaceutically acceptable carrier.

50. The pharmaceutical composition of claim 49, wherein the carrier is a diluent, an aerosol, a topical carrier, an aqueous solution, a nonaqueous solution or a solid carrier.

51. An isolated nucleic acid which encodes a wildtype human Beclin.

52. An isolated nucleic acid which encodes a mutant human Beclin.

53. The isolated nucleic acid of claim 51 or 52, wherein the nucleic acid is DNA.

54. The isolated nucleic acid of claim 51 or 52, wherein the nucleic acid is RNA.

55. The isolated nucleic acid of claim 53, wherein the nucleic acid is cDNA.

56. The isolated nucleic acid of claim 53, wherein the nucleic acid is genomic DNA.

57. The isolated nucleic acid of claim 53, wherein the wildtype Beclin has an amino acid sequence substantially identical to the amino acid sequence designated Seq. I.D. No.: 1A.

58. The isolated nucleic acid of claim 53, wherein the nucleic acid comprises a nucleic acid having a sequence substantially the same as the sequence designated Seq. I.D. No.: 1B.

59. An isolated wildtype human beclin protein.

60. An isolated mutant human beclin protein.

61. The protein of claim 59, having the same amino acid sequence as the amino acid sequence designated Seq. I.D. No.: 1A.

62. The isolated nucleic acid of claim 59, having the same amino acid sequence as the amino acid sequence designated Seq. I.D. No.: 1B.

63. A vector comprising the nucleic acid of claim 51 operatively linked to a promoter of RNA transcription.

64. The vector of claim 63, wherein the promoter comprises
     a bacterial, yeast, insect or mammalian promoter.

5 65. The vector of claim 63, further comprising plasmid,
        cosmid,   yeast   artificial   chromosome   (YAC),
        bacteriophage or eukaryotic viral DNA.

66. The plasmid of claim 65 designated pSG5/beclin (ATCC
10   Accession Number 97664).

67. A host vector system for the production of a
     polypeptide which comprises the vector of claim 63 in
     a suitable host.

15
68. The host vector system of claim 63, wherein the
     suitable host comprises a prokaryotic or eukaryotic
     cell.

20 69. The host vector system of claim 63, wherein the
        prokaryotic cell comprises a bacterial cell.

70. The host vector system of claim 63, wherein the
     eukaryotic cell comprises an yeast, insect, plant or
25   mammalian cell.

71. A method of producing a polypeptide which comprises
     growing the host vector system of claim 63, under
     suitable conditions permitting production of the
30   polypeptide and recovering the polypeptide so produced.

72. A method of obtaining a polypeptide in purified form
     which comprises:

35   (a) introducing the vector of claim 63 into a suitable
             host cell;
         (b) culturing the resulting host cell so as to produce
             the polypeptide;

(c) recovering the polypeptide produced into step (b);
     and

(d) purifying the polypeptide so recovered.

73. The method of claim 72, wherein the vector comprises
     plasmid, cosmid, yeast artificial chromosome (YAC),
     bacteriophage or eukaryotic viral DNA.

74. The method of claim 72, wherein the suitable host cell
     comprises a bacterial, insect, plant or mammalian cell.

75. A purified, wildtype human Beclin.

76. An oligonucleotide of at least 15 nucleotides capable
     of specifically hybridizing with a unique sequence of
     nucleotides within a nucleic acid which encodes a
     wildtype Beclin without hybridizing to any sequence of
     nucleotides within a nucleic acid which encodes a
     mutant human Beclin.

77. An oligonucleotide of at least 15 nucleotides capable
     of specifically hybridizing with a unique sequence of
     nucleotides within a nucleic acid which encodes a
     mutant Beclin without hybridizing to any sequence of
     nucleotides within a nucleic acid which encodes a
     wildtype human Beclin.

78. The oligonucleotide of claim 76 or claim 77, wherein
     the nucleic acid is DNA.

79. The oligonucleotide of claim 76 or claim 77, wherein
     the nucleic acid is RNA.

80. A method for determining whether a subject has a
     predisposition for cancer which comprises:

     (a) obtaining an appropriate nucleic acid sample from
     the subject; and

(b) determining whether the nucleic acid sample from
step (a) is, or is derived from, a nucleic acid which
encodes a mutant human Beclin so as to thereby
determine whether a subject has a predisposition for
cancer.

81.   The method of claim 80, wherein the nucleic acid sample
in step (a) comprises mRNA corresponding to the
transcript of DNA encoding a mutant Beclin, and wherein
the determining of step (b) comprises:

(i) contacting the mRNA with the oligonucleotide of
claim 23 under conditions permitting binding of the
mRNA to the oligonucleotide so as to form a complex;
(ii) isolating the complex so formed; and
(iii)identifying the mRNA in the isolated complex so as
to thereby determine whether the mRNA is, or is derived
from, a nucleic acid which encodes a mutant human
Beclin.

82.   The method of claim 80, wherein the determining of step
(b) comprises:

(i) contacting the nucleic acid sample of step (a), and
the isolated nucleic acid of claim 1 with restriction
enzymes under conditions permitting the digestion of
the nucleic acid sample, and the isolated nucleic acid
into distinct, distinguishable pieces of nucleic acid;
(ii) isolating the pieces of nucleic acid; and
(iii) comparing the pieces of nucleic acid derived from
the nucleic acid sample with the pieces of nucleic acid
derived from the isolated nucleic acid so as to thereby
determine whether the nucleic acid sample is, or is
derived from, a nucleic acid which encodes a mutant
human Beclin.

83.   The method of claim 80, wherein the determining of step
(b) comprises:

                (i)   sequencing the nucleic acid sample of step (a);
                      and
                (ii) comparing the nucleic acid sequence of step (i)
5   with the isolated nucleic acid of claim 62, so as to
                thereby determine whether the nucleic acid sample is,
                or is derived from, a nucleic acid which encodes a
                mutant human Beclin.

10   84.   The method of claim 81, wherein the nucleic acid sample
            in step (a) comprises mRNA corresponding to the
            transcript of DNA encoding mutant Beclin, and wherein
            the determining of step (b) comprises:

15   (i) translating the mRNA under suitable conditions to
                obtain an amino acid sequence; and
                (ii) comparing the amino acid sequence of step (i) with
                the isolated nucleic acid of claim 7 so as to thereby
                determine whether the nucleic acid sample is, or is
20   derived from, a nucleic acid which encodes a mutant
                human Beclin.

     85.   The method of claim 81, wherein the determining of step
            (b) comprises:

25
                (i) amplifying the nucleic acid present in the sample
                of step (a); and
                (ii) detecting the presence of the mutant human Beclin
                in the resulting amplified nucleic acid.

30
     86.   The method of claim 80 or claim 81, wherein the
            isolated nucleic acid or the oligonucleotide is labeled
            with a detectable marker.

35   87.   The method the claim 86, wherein the detectable marker
            is a radioactive isotope, a fluorophor or an enzyme.

88.   The method of claim 86, wherein the sample comprises
       blood, tissue or sera.

89.   A method for determining whether a subject has cancer,
5   which comprises:

       (a) obtaining an appropriate nucleic acid sample from
       the subject; and
       (b) determining whether the nucleic acid sample from
10   step (a) is, or is derived from, a nucleic acid which
       encodes a mutant human Beclin so as to thereby
       determine whether a subject has cancer.

90.   The method of claim 89, wherein the nucleic acid sample
15   in step (a) comprises mRNA corresponding to the
       transcript of DNA encoding a mutant Beclin, and wherein
       the determining of step (b) comprises:

       (i) contacting the mRNA with the oligonucleotide of
20   claim 23 under conditions permitting binding of the
       mRNA to the oligonucleotide so as to form a complex;
       (ii) isolating the complex so formed; and
       (iii)identifying the mRNA in the isolated complex so as
       to thereby determine whether the mRNA is, or is derived
25   from, a nucleic acid which encodes a mutant human
       Beclin.

91.   The method of claim 90, wherein the determining of step
       (b) comprises:
30   (i) contacting the nucleic acid sample of step (a), and
       the isolated nucleic acid of claim 1 with restriction
       enzymes under conditions permitting the digestion of
       the nucleic acid sample, and the isolated nucleic acid
       into distinct, distinguishable pieces of nucleic acid;
35   (ii) isolating the pieces of nucleic acid; and
       (iii) comparing the pieces of nucleic acid derived from
       the nucleic acid sample with the pieces of nucleic acid
       derived from the isolated nucleic acid so as to thereby

determine whether the nucleic acid sample is, or is
derived from, a nucleic acid which encodes a mutant
human Beclin.

92. The method of claim 90, wherein the determining of step
     (b) comprises:

     (i)   sequencing the nucleic acid sample of step (a);
           and
     (ii) comparing the nucleic acid sequence of step (i)
     with the isolated nucleic acid of claim 8, so as to
     thereby determine whether the nucleic acid sample is,
     or is derived from, a nucleic acid which encodes a
     mutant human Beclin.

93. The method of claim 90, wherein the nucleic acid sample
     in step (a) comprises mRNA corresponding to the
     transcript of DNA encoding a mutant Beclin, and wherein
     the determining of step (b) comprises:
     (i) translating the mRNA under suitable conditions to
     obtain an amino acid sequence; and
     (ii) comparing the amino acid sequence of step (i) with
     the isolated nucleic acid of claim 7 so as to thereby
     determine whether the nucleic acid sample is, or is
     derived from, a nucleic acid which encodes a mutant
     human Beclin.

94. The method of claim 90, wherein the determining of step
     (b) comprises:

     (i) amplifying the nucleic acid present in the sample
     of step (a); and
     (ii) detecting the presence of the mutant human Beclin
     in the resulting amplified nucleic acid.

95. The method of claim 94 or claim 95, wherein the
     isolated nucleic acid or the oligonucleotide is labeled
     with a detectable marker.

96. The method the claim 89, wherein the detectable marker
     is a radioactive isotope, a fluorophor or an enzyme.

5 97. The method of claim 89, wherein the sample comprises
        blood, tissue or sera.

98. A method for inhibiting cell proliferation in cells
     unable   to   regulate   themselves   which   comprises
10   introducing the isolated nucleic acid of claim 1 into
     the cells.

99. The method of claim 97, wherein the cells are
     cancerous.
15

100. A method of treating a subject who has cancer which
      comprises introducing the isolated nucleic acid of
      claim 51 or 52, into the subject so as to thereby treat
      the cancer.
20

101. The method of claim 99, wherein introducing the
      isolated nucleic acid, comprises:

      (a) recovering cancer cells from the subject;
25   (b) introducing the isolated nucleic acid of claim 1
           into the cells; and
      (c) reintroducing the cells of step (b) into the
           subject so as to treat the subject who has cancer.

30 102. The method of claim 99, wherein the cancer cells are
          breast, ovarian, skeletal, cervical, colon, prostate or
          lung cells.

103. A pharmaceutical composition comprising a wildtype
35   human Beclin and a pharmaceutically acceptable carrier.

104. A pharmaceutical composition comprising the polypeptide
     obtained from the method of claim 18 and a
     pharmaceutically acceptable carrier.

5 105. A method for treating a subject who has cancer
        comprising administration of an effective amount of the
        pharmaceutical composition of claim 102 and claim 103
        to the subject who has cancer.

FIG. 1A

```
      S  E  S  G  T  L  E  Q  F  W  L  F  F  N  K
      D  T  E  M  M  V  D  Q  A  Q  V  E  R  R  F  N
      D  E  Q  M  V  L  D  E  V  R  N  V  R  L  T  Q  F  Y
      L  T  R  K  Q  S  I  K  T  P  R  Q  L  E  T  Q  F
      K  E  G  A  R  L  D  D  K  F  K  T  Q  G  G  K  I  K  T  Q
      L  P  G  P  R  L  E  D  D  R  K  E  F  G  G  K  S  I  S
      Q  P  K  P  P  R  L  E  K  N  S  L  P  L  K  S  V  E  Y  S  S
      Q  K  I  I  S  N  N  E  K  E  Y  K  L  L  C  L  Y  S  Y  S  S
      S  A  K  F  L  D  T  D  M  N  E  B  E  Y  D  K  L  Y  C  E  S  Y  S  W
      C  Q  R  R  E  N  L  T  E  Q  D  V  R  Q  L  G  R  K  M  E  G  G  L  A
      R  A  Q  R  E  C  L  E  D  Q  Y  Q  L  R  L  G  K  N  P  F  K  G  G  L
      Q  T  T  S  V  M  E  E  L  L  E  D  Y  T  Q  R  A  R  N  L  E  P  F  K  W
      C  V  L  T  G  V  C  E  E  L  I  L  E  A  Q  Y  N  F  A  K  E  Q  G  G  W
      P  V  L  D  G  G  C  E  E  I  L  Q  E  L  A  Q  Y  A  N  A  L  A  K  E  Q  Q  G
      F  L  A  D  D  D  P  L  C  I  L  O  E  E  R  Y  I  N  A  K  S  K  C  V  V  G
      S  P  R  Q  D  D  G  P  L  C  L  O  E  E  R  I  H  A  D  K  S  C  C  V  E  D  T  N  L
      V  A  P  R  V  S  D  H  D  K  R  C  L  D  Q  M  R  T  I  L  D  D  L  C  E  D  T  L  T  N
      Q  T  T  P  E  V  D  D  H  K  R  E  R  L  D  D  Q  O  F  G  T  L  L  T  L  F  L  K  I  E  L
      M  L  T  E  T  G  E  D  V  Y  K  E  R  L  R  E  D  Q  G  F  V  L  S  D  A  F  L  K  F
      S  Q  I  I  G  T  D  N  Q  E  Q  E  B  A  E  R  E  S  V  G  Q  T  L  E  S  A  M  K  G  F
      N  I  F  L  L  Q  C  Q  B  A  L  A  E  E  K  S  S  Q  L  E  S  M  V  E  B  K  G
      N  T  P  F  T  G  E  C  L  A  E  E  L  K  S  H  G  Y  L  A  A  V  D  B  K  A
      S  V  E  F  L  S  G  N  E  L  A  K  W  W  H  S  D  H  A  D  K  A
      K  T  D  R  E  E  S  M  I  N  E  B  K  E  V  Q  E  L  H  I  A  A  N  H  S  F  D  R  M  T  K
      K  L  D  R  G  N  I  T  K  K  V  D  D  H  A  G  N  K  F  Y  R
      S  L  N  S  A  P  D  I  N  V  E  L  B  K  D  D  F  N  G  K  F  P  Y  R  Q
      G  I  N  S  A  D  L  F  N  E  L  B  L  B  L  A  T  I  P  Y  D  N  L  P  Y  E  E
      E  K  L  T  E  L  D  L  N  E  I  L  A  P  E  I  N  E  I  V  W  D  N  C  L  P  E  E
      M  F  E  T  E  D  Q  L  Q  N  E  L  A  E  N  L  Z  V  W  C  W  S  K  K  A

      1  31 61 91 121 151 181 211 241 271 301 331 361 391 421
```

1/1
ATG GAA GGG TCT AAG AGC AGC ACC ATG GTG ACC TTC AAC CAG GTG TCC TCA ACC AAC TCC AAC CTG AAA CTG GAC ACG ATT
M   E   G   S   K   S   S   T   M   V   T   F   N   Q   V   S   S   T   N   S   N   L   K   L   D   T   I   S
91/31   31/11
TTC AAG ATC CTG GAC CTT CTC ACC ATC CAG GAA CTC ACA GCT CCA TTA CTT ACC ATC ACA GCC CAG GCG AAA CTA GTA GAC CAT GAG GAA
F   K   I   L   D   L   L   T   I   Q   E   L   T   A   P   L   L   T   I   T   A   Q   A   K   L   V   D   H   E   E
181/61   121/41
GAG ACT AAC TAC TCA GGA GAG GAG CCA TTT ATT GAA ACT GAC TTC ATC ATC CCA ACA TTC ACT CCC CCA GCC GCC AGG ATT ATT TCC
E   T   N   Y   S   G   E   E   P   F   I   E   T   D   F   I   I   P   T   F   T   P   P   A   A   R   I   I   S
271/91   211/71
ACA GAA AGT GCC AAC AGC TCT ACT TTC ATT GGG GAG GTA TCT GAT GGC GGC ACC ATG GAG AAC CTC AGC CTA ACA CTG AAG GTC ACT GTC
T   E   S   A   N   S   S   T   F   I   G   E   V   S   D   G   G   T   M   E   N   L   S   L   T   L   K   V   T   V
361/121   301/101
GAC CTG TTT GAC ATC ATG TCG GAT GTG GAT GAC CAC ACA GAT GTG ACA TAC ACA GAT TAC CAC CTC CTC GAC ACT
D   L   F   D   I   M   S   D   V   D   D   H   P   L   C   S   R   R   L   L   D   T   T   H   E   T
451/151   391/131
CAC CTC AAC GTC ACT GAA AAT GAG TGT CAG AAA TAC TAC AAG AAG AAG AAC AAT AGG CAA TGG
H   L   N   V   T   E   N   E   C   Q   K   Y   Y   K   R   K   N   N   R   Q   W
541/181 421/141
CAG ATG GAG CTA AAG GAG CTG GCA CTA AAG GAG ATC CTG ATC CAG GAG CTG CAA CAA ATG GTG TCG GAA
Q   M   E   L   K   E   L   A   L   K   E   I   L   I   Q   E   L   Q   Q   M   V   S   E
631/211 511/171
AAT GTC GAG GAT GAT GAG CTG GCT CAG CTC CGG AGA CTG ATT AAT AAC TTC ACG CTG GAT AAT
N   V   E   D   D   E   L   A   Q   L   R   R   L   I   N   N   F   T   L   D   N
721/241 601/201
CTG GAG CTG GAT GAT GAG CTG AAG GAG CTG CAG CTG ATC CAG AAC CTG GAG AGA CAG GCT CAG CTG
L   E   L   D   D   E   L   K   E   L   Q   L   I   Q   N   L   E   R   Q   A   Q   L
811/271 691/231
AAT GCA ACC TTC TTC ATC ATG TGG CAG AGT GGA CAG TTT GGC ACA ATC AAT AAT AAC TTC AGG CTG GAT
N   A   T   F   F   I   M   W   Q   S   G   Q   F   G   T   I   N   N   N   F   R   L   D
901/301 781/261
AAT CAG ATT AAT GTG GAC ATC CAT TCA TAT CTG TTG TTC CTG CTG GAC AAA TCT AAG GAG CTG AAA
N   Q   I   N   V   D   I   H   S   Y   L   L   F   L   L   D   K   S   K   E   L   K
991/331 871/291
CTT CCT TAC GGA AAC CAT TCA GCT CCA TAT CTG TTC GGT TTC GGT CTG AAA CTG TAC AAG GAG GGT CCG
L   P   Y   G   N   H   S   A   P   Y   L   F   G   F   G   L   K   L   Y   K   E   G   P
1081/361 961/321
TGG GAC AAC AAG ATT GAC TTT GAC CAT GCA ATG GTC GAG AAA GAG ATC AGC GAC GTC CTT GAT
W   D   N   K   I   D   F   D   H   A   M   V   E   K   E   I   S   D   V   L   D
1171/391 1051/351
TGT CTT CCC TAC CTG GAC GGG ACA TGG AGA TTC TTC CAC CTG ACG TGT GGT TTC GGA
C   L   P   Y   L   D   G   T   W   R   F   F   H   L   T   C   G   F   G
1261/421 1141/381
TCT GAG GAC GAG TGG GAC TTC ATG GTG GAA TTC CTG AAA TGG TTA ATC AAA
S   E   D   E   W   D   F   M   V   E   F   L   K   W   L   I   K
1351/451 1231/411
TGA
*

FIG. 1B

FIG. 2A

FIG. 2B

FIG. 2D

FIG. 2C

FIG. 3C

FIG. 3B

FIG. 3A

FIG. 3F

FIG. 3E

FIG. 3D

FIG. 3G

FIG. 3H

FIG. 3I

FIG. 4A

FIG. 4B

FIG. 4C

NIH 3T3 clone

FIG. 5A

FIG. 5B

FIG. 5C

FIG. 6A

FIG. 6B

FIG. 6C

FIG. 7

FIG. 8A Beclin 5' UTR - clone 5

```
cgggagtcgctgaagacgagcagcgatggtagttctggaggcctgctcccgggccgaccc
gaggccacagtgcctccgcgggtagacgggctgggtgacgggctccgggctcccgaggg
```

FIG. 8B Beclin 3' UTR - clone 13

```
cttttcctagggggaggttgcctaaaggcttttaatttgttttgttgttgcaaac
atgtttaaattaaattcgggtaatattaaacagtacatgttacaataccaaaaagaa
aaatccacaaaagccacttattttaaaatatcatgtgacagatacttccagagctac
aacatgccatctatagttgcagcccctggtcagtttgatcctaaccccatggactcct
ttcccttcttctctgaaaaaactaattaaattgcttttcttttttaactgagtt
gaatgagattgatgtgtttcactggattttatctcctccaactcctgcacttaaca
atatgaatagaaactttgtccttactgagatgggatatgtttgagatgcacagttgg
ataatgtgggaaaatgacatccaagcttacctggtcaccatgtgatgtgtcagatgct
tgaaattaacacttcacttggttcctatactgaatgccgactctgctcttgttga
gatatgaaatggtgttgatactgtttgagacattatgagagattaattattgtaat
aaagatttgctgcgcagtctgaaaacggaaaaaaaaaaaaaaaaaaaaa
```

FIG. 9A

FIG. 9B

FIG. 9C

FIG. 9D

FIG. 9E

FIG. 10A

FIG. 10B

21/41

FIG. 11

FIG. 12A   FIG. 12B

FIG. 13

```
  1  MEGSKTSNNS TMQVSFVCQR CSQPLKLDTS
 31  FKILDRVTIQ ELTAPLLTTA QAKPGETQEE
 61  ETNSGEEPFI ETPRQDGVSR RFIPPARMMS
 91  TESANSFTLI GEASDGGTME NLSRRLKVTG
121  DLFDIMSGQT DVDHPLCEEC TDTLLDQLDT
151  QLNVTENECQ NYKRCLEILE QMNEDDSEQL
181  QMELKELALE ERLIQELEDV EKNRKIVAE
211  NLEKVQAEAE RLDQEEAQYQ REYSEFKRQQ
241  LELDDELKSV ENQMRYAQTQ LDKLKKTNVF
271  NATFHIWHSG QFGTINNFRL GRLPSVPVEW
301  NEINAAWGQT VLLHALANKM GLKFQRYRL
331  VPYGNHSYLE SLTDKSKELP LYCSGGLRFF
361  WDNKFDHAMV AFLDCVQQFK EEVEKGETRF
391  CLPYRMDVEK GKIEDTGGSG GSYSIKTQFN
421  SEEQWTKALK FMLTNLKWGL AWVSSQFYNK
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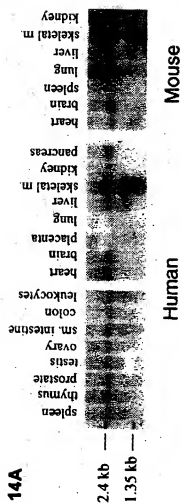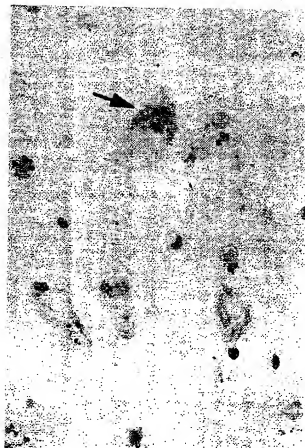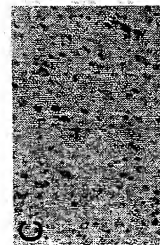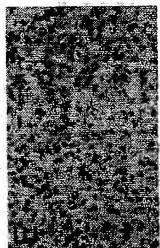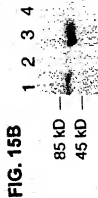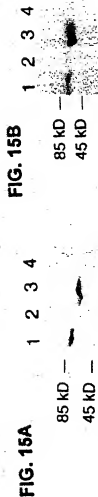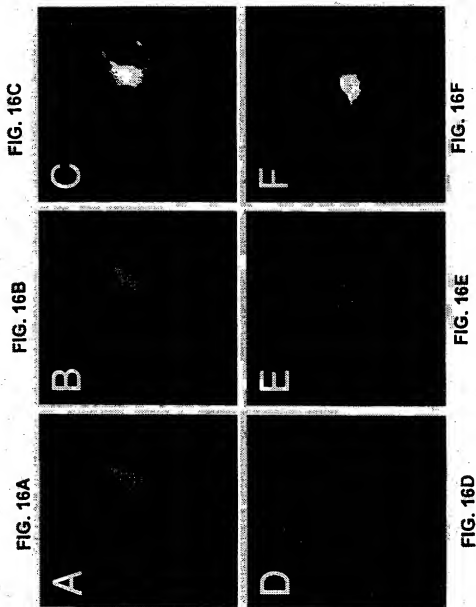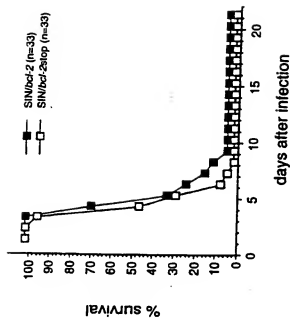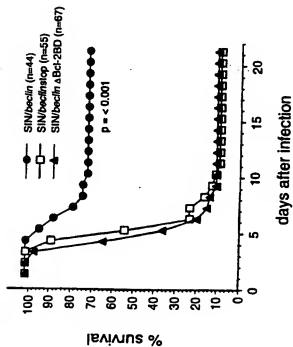
FIG. 14A

FIG. 14B

FIG. 15A

85 kD —
45 kD —

1 2 3 4

FIG. 15B

85 kD —
45 kD —

1 2 3 4

FIG. 15C

FIG. 15D

FIG. 15E

FIG. 16A FIG. 16B FIG. 16C FIG. 16D FIG. 16E FIG. 16F

FIG. 17B

FIG. 17A

FIG. 18

FIG. 19

FIG. 20

FIG. 21A

FIG. 21B

FIG. 21C

FIG. 21D

FIG. 21E

FIG. 21F

FIG. 22

γ HV68 Growth

FIG. 23B

(−) PMSF   (+) PMSF

SEY6210

JCY3000

JCY3000 + pRS424

JCY3000 + pRS424 *vps30*

JCY3000 + pRS424 *beclin1*

FIG. 23A

flag-Beclin 1 —

1   2   3   4   5   6

FIG. 23C

FIG. 24B

FIG. 24D

b

d

a

c

FIG. 24A

FIG. 24C

FIG. 24E

FIG. 24F

FIG. 25B

FIG. 25A

FIG. 25D

FIG. 25C

FIG. 25E

FIG. 25F

FIG. 26A

Beclin 1 —

Actin —

FIG. 26B   441 458 386 526 577 341 246
            C N T N T N T N T N T N T N T

Beclin 1 —

Actin —

FIG. 26C   441N   441T

           526N   526T
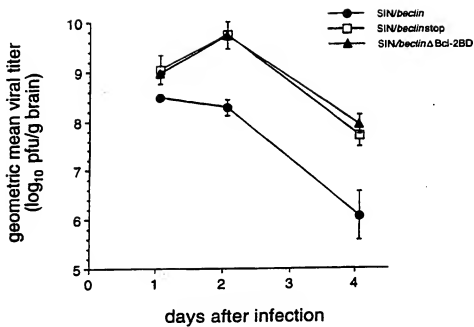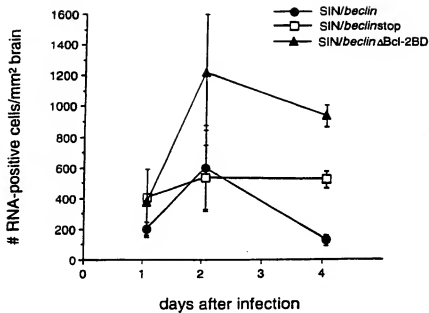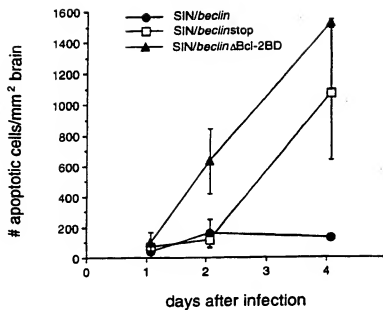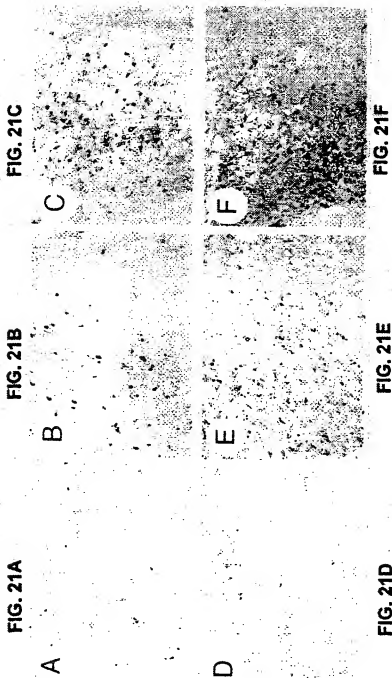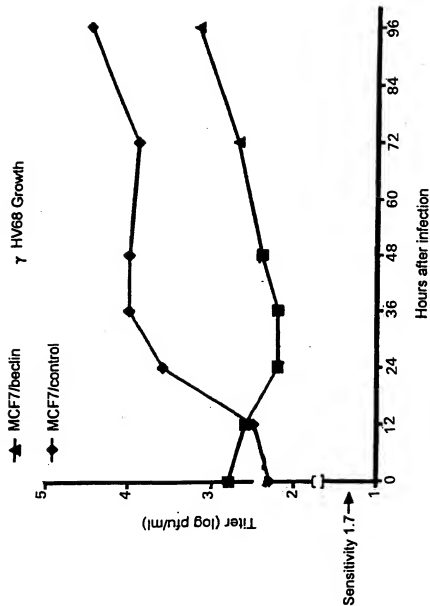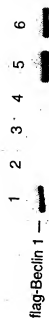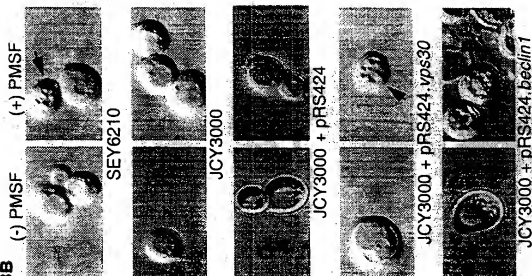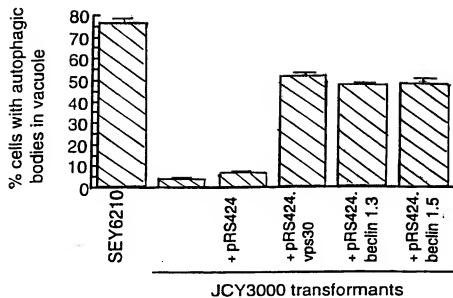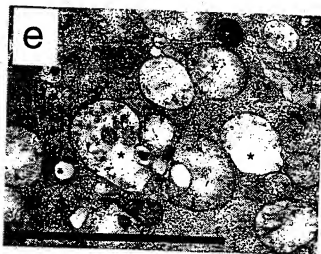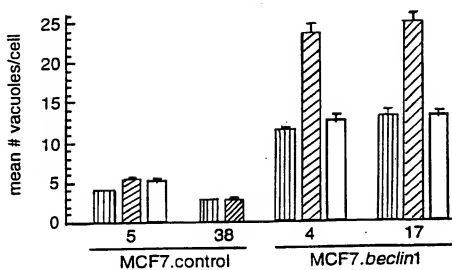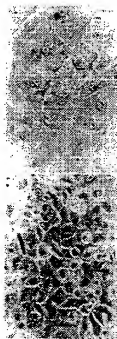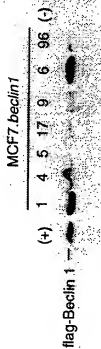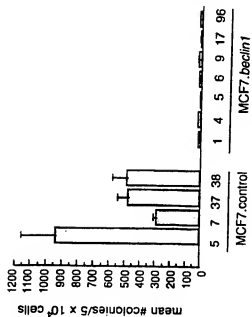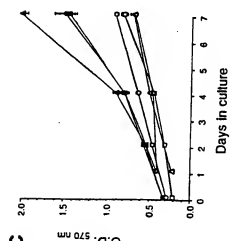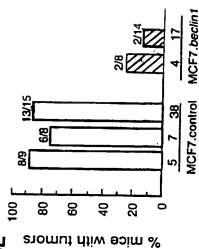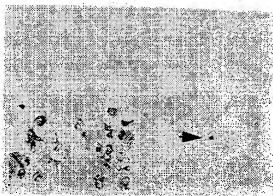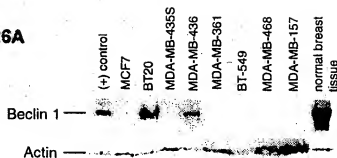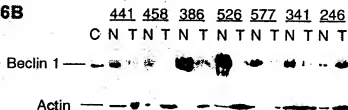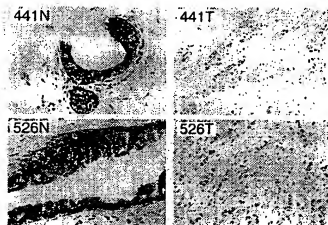
SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Levine, Beth C.

    (ii) TITLE OF INVENTION: BECLIN AND USES THEREOF

    (iii) NUMBER OF SEQUENCES: 22

    (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Cooper & Dunham LLP
        (B) STREET: 1185 Avenue of the Americas
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        (D) STATE: New York
        (E) COUNTRY: USA
        (F) ZIP: 10036

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: Not Yet Known
        (B) FILING DATE: 9 March 1999
        (C) CLASSIFICATION:

   (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: White, John P
        (B) REGISTRATION NUMBER: 28,678
        (C) REFERENCE/DOCKET NUMBER: 50902-B/JPW/AMG

    (ix) TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: (212) 278-0400
        (B) TELEFAX: (212) 391-0525

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 450 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

   (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu Gly Ser Lys Thr Ser Asn Asn Ser Thr Met Gln Val Ser Phe
1   5   10   15

Val Cys Gln Arg Cys Ser Gln Pro Leu Lys Leu Asp Thr Ser Phe Lys
            20   25   30

Ile Leu Asp Arg Val Thr Ile Gln Glu Leu Thr Ala Pro Leu Leu Thr
        35   40   45

Thr Ala Gln Ala Lys Pro Gly Glu Thr Gln Glu Glu Glu Thr Asn Ser
    50   55   60

Gly Glu Glu Pro Phe Ile Glu Thr Pro Arg Gln Asp Gly Val Ser Arg
65   70   75   80

Arg Phe Ile Pro Pro Ala Arg Met Met Ser Thr Glu Ser Ala Asn Ser
                85   90   95

Phe Thr Leu Ile Gly Glu Val Ser Asp Gly Gly Thr Met Glu Asn Leu
            100   105   110

Ser Arg Arg Leu Lys Val Thr Gly Asp Leu Phe Asp Ile Met Ser Gly
        115   120   125

Gln Thr Asp Val Asp His Pro Leu Cys Glu Glu Cys Thr Asp Thr Leu
    130   135   140

Leu Asp Gln Leu Asp Thr Gln Leu Asn Val Thr Glu Asn Glu Cys Gln
145   150   155   160

Asn Tyr Lys Arg Cys Leu Glu Ile Leu Glu Gln Met Asn Glu Asp Asp
                165   170   175

Ser Glu Gln Leu Gln Met Glu Leu Lys Glu Leu Ala Leu Glu Glu Glu
            180   185   190

Arg Leu Ile Gln Glu Leu Glu Asp Val Glu Lys Asn Arg Lys Ile Val
        195   200   205

Ala Gln Asn Leu Glu Lys Val Gln Ala Glu Ala Glu Arg Leu Asp Gln
    210   215   220

Glu Glu Ala Gln Tyr Gln Arg Glu Tyr Ser Glu Phe Lys Arg Gln Gln
225   230   235   240

Leu Glu Leu Asp Asp Glu Leu Lys Ser Val Glu Asn Gln Met Arg Tyr
            245   250   255

Ala Gln Thr Gln Leu Asp Lys Leu Lys Lys Thr Asn Val Phe Asn Ala
        260   265   270

Thr Phe His Ile Trp His Ser Gly Gln Phe Gly Thr Ile Asn Asn Phe
        275   280   285

2

Arg Leu Gly Arg Leu Pro Ser Val Pro Val Glu Trp Asn Glu Ile Asn
    290   295   300

Ala Ala Trp Gly Gln Thr Val Leu Leu Leu His Ala Leu Ala Asn Lys
305   310   315   320

Met Gly Leu Lys Phe Gln Arg Tyr Arg Leu Val Pro Tyr Gly Asn His
                325   330   335

Ser Tyr Leu Glu Ser Leu Thr Asp Lys Ser Lys Glu Leu Pro Leu Tyr
            340   345   350

Cys Ser Gly Gly Leu Arg Phe Phe Trp Asp Asn Lys Phe Asp His Ala
            355   360   365

Met Val Ala Phe Leu Asp Cys Val Gln Gln Phe Lys Glu Glu Val Glu
    370   375   380

Lys Gly Glu Thr Arg Phe Cys Leu Pro Tyr Arg Met Asp Val Glu Lys
385   390   395   400

Gly Lys Ile Glu Asp Thr Gly Gly Ser Gly Gly Ser Tyr Ser Ile Lys
                405   410   415

Thr Gln Phe Asn Ser Glu Glu Gln Trp Thr Lys Ala Leu Lys Phe Met
            420   425   430

Leu Thr Asn Leu Lys Trp Gly Leu Ala Trp Val Ser Ser Gln Phe Tyr
        435   440   445

Asn Lys
    450

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1353 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (iii) HYPOTHETICAL: NO

   (iv) ANTI-SENSE: NO

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGGAAGGGT CTAAGACGTC CAACAACAGC ACCATGCAGG TGAGCTTCGT GTGCCAGCGC   60

TGCAGCCAGC CCCTGAAACT GGACACGAGT TTCAAGATCC TGGACCGTGT CACCATCCAG   120

GAACTCACAG CTCCATTACT TACCACAGCC CAGGCGAAAC CAGGAGAGAC CCAGGAGGAA   180

GAGACTAACT CAGGAGAGGA GCCATTTATT GAAACTCCTC GCCAGGATGG TGTCTCTCGC   240

AGATTCATCC CCCCAGCCAG GATGATGTCC ACAGAAAGTG CCAACAGCTT CACTCTGATT   300

GGGGAGGTAT CTGATGGCGG CACCATGGAG AACCTCAGCC GAAGACTGAA GGTCACTGGG   360

GACCTTTTTG ACATCATGTC GGGCCAGACA GATGTGGATC ACCCACTCTG TGAGGAATGC   420

ACAGATACTC TTTTAGACCA GCTGGACACT CAGCTCAACG TCACTGAAAA TGAGTGTCAG   480

AACTACAAAC GCTGTTTGGA GATCTTAGAG CAAATGAATG AGGATGACAG TGAACAGTTA   540

CAGATGGGAGC TAAAGGAGCT GGCACTAGAG GAGGAGAGGC TGATCCAGGA GCTGGAAGAC   600

GTGGAAAAGA ACCGCAAGAT AGTGGCAGAA AATCTCGAGA AGGTCCAGGC TGAGGCTGAG   660

AGACTGGATC AGGAGGAAGC TCAGTATCAG AGAGAATACA GTGAATTTAA ACGACAGCAG   720

CTGGAGCTGG ATGATGAGCT GAAGAGTGTT GAAAACCAGA TGCGTTATGC CCAGACGCAG   780

CTGGATAAGC TGAGAAAAAC CAACGTCTTT AATGCAACCT TCCACATCTG GCACAGTGGA   840

CAGTTTGGCA CAATCAATAA CTTCAGGCTG GGTCGCCTGC CCAGTGTTCC CGTGGAATGG   900

AATGAGATTA ATGCTGCTTG GGGCCAGACT GTGTTGCTGC TCCATGCTCT GGCCAATAAG   960

ATGGGTCTGA AATTTCAGAG ATACCGACTT GTTCCTTACG GAAACCATTC ATATCTGGAG   1020

TCTCTGACAG ACAAATCTAA GGAGCTGCCG TTATACTGTT CTGGGGGGTT GCGGTTTTTC   1080

TGGGACAACA AGTTTGACCA TGCAATGGTG GCTTTCCTGG ACTGTGTGCA GCAGTTCAAA   1140

GAAGAGGTTG AGAAAGGCGA GACACGTTTT TGTCTTCCCT ACAGGATGGA TGTGGAGAAA   1200

GGCAAGATTG AAGACACAGG AGGCAGTGGC GGCTCCTATT CCATCAAAAC CCAGTTTAAC   1260

TCTGAGGAGC AGTGGACAAA AGCTCTCAAG TTCATGCTGA CGAATCTTAA GTGGGGTCTT   1320

GCTTGGGTGT CCTCACAATT TTATAACAAA TGA   1353

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 120 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: not relevant

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGGGAAGTCG CTGAAGACAG AGCGATGGTA GTTCTGGAGG CCTCGCTCCG GGGCCGACCC   60

GAGGCCACAG TGCCTCCGCG GTAGACCGGA CTTGGGTGAC GGGCTCCGGG CTCCCGAGGG   120

(2) INFORMATION FOR SEQ ID NO:4:

     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 654 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: not relevant
         (D) TOPOLOGY: not relevant

     (ii) MOLECULE TYPE: DNA (genomic)

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTTTTTTCCT TAGGGGGAGG TTTGCCTTAA AGGCTTTTTA ATTTTGTTTT GTTTGCAAAC   60

ATGTTTTAAA TTAAATTCGG GTAATATTAA ACAGTACATG TTTACAATAC CAAAAAAGAA   120

AAAATCCACA AAAGCCACTT TATTTTAAAA TATCATGTGA CAGATACTTT CCAGAGCTAC   180

AACATGCCAT CTATAGTTGC CAGCCCTGGT CAGTTTTGAT TCTTAACCCC ATGGACTCCT   240

TTCCCTTTCT TCTCTGAAAA AAACTAATTT AAATTTGCTT TTCTTTTTTT TAACTGAGTT   300

GAATTGAGAT TGATGTGTTT TCACTGGATT TTTATCTCTC TCAACTTCCT GCACTTAACA   360

ATATGAAATA GAAACTTTTG TCTTTACTGA GATGAGGATA TGTTTGAGAT GCACAGTTGG   420

ATAATGTGGG AAAATGACAT CTAAGCTTTA CCTGGTCACC ATGTGATGTG ATCAGATGCT   480

TGAAATTTAA CACTTTTCAC TTGGTTCTTA TACTGAATGC CGACTCTGCT CTGTGTTAGA   540

GATATGAAAT GGTGTTTGAT ACTGTTTGAG ACATTATGGA GAGATTTAAT TATTTGTAAT   600

AAAAGATTTG CTGCAGTCTG AAAACCGGAA AAAAAAAAA AAAAAAAAA AAAA   654

(2) INFORMATION FOR SEQ ID NO:5:

     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 1383 base pairs

                                    5

```
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: not relevant
            (D) TOPOLOGY: not relevant

      (ii) MOLECULE TYPE: DNA (genomic)




      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGGAAGGGT CTAAGACGTC CAACAACAGC ACCATGCAGG TGAGCTTCGT GTGCCAGCGC        60

TGCAGCCAGC CCCTGAAACT GGACACGAGT TTCAAGATCC TGGACCGTGT CACCATCCAG       120

GAACTCACAG CTCCATTACT TACCACAGCC CAGGCGAAAC CAGGAGAGAC CCAGGAGGAA       180

GAGACTAACT CAGGAGAGGA GCCATTTATT GAAACTCCTC GCCAGGATGG TGTCTCTCGC       240

AGATTCATCC CCCCAGCCAG GATGATGTCC ACAGAAAGTG CCAACAGCTT CACTCTGATT       300

GGGGAGGCAT CTGATGGCGG CACCATGGAG AACCTCAGCC GAAGACTGAA GGTCACTGGG       360

GACCTTTTTG ACATCATGTC GGGCCAGACA GATGTGGATC ACCCACTCTG TGAGGAATGC       420

ACAGATACTC TTTTAGACCA GCTGGACACT CAGCTCAACG TCACTGAAAA TGAGTGTCAG       480

AACTACAAAC GCTGTTTGGA GATCTTAGAG CAAATGAATG AGGATGACAG TGAACAGTTA       540

CAGATGGAGC TAAAGGAGCT GGCACTAGAG GAGGAGAGGC TGATCCAGGA GCTGGAAGAC       600

GTGGAAAAGA ACCGCAAGAT AGTGGCAGAA AATCTCGAGA AGGTCCAGGC TGAGGCTGAG       660

AGACTGGATC AGGAGGAAGC TCAGTATCAG AGAGAATACA GTGAATTTAA ACGACAGCAG       720

CTGGAGCTGG ATGATGAGCT GAAGAGTGTT GAAAACCAGA TGCGTTATGC CCAGACGCAG       780

CTGGATAAGC TGAAGAAAAC CAACGTCTTT AATGCAACCT TCCACATCTG GCACAGTGGA       840

CAGTTTGGCA CAATCAATAA CTTCAGGCTG GGTCGCCTGC CCAGTGTTCC CGTGGAATGG       900

AATGAGATTA ATGCTGCTTG GGGCCAGACT GTGTTGCTGC TCCATGCTCT GGCCAATAAG       960

ATGGGTCTGA AATTTCAGAG ATACCGACTT GTTCCTTACG GAAACCATTC ATATCTGGAG      1020

TCTCTGACAG ACAAATCTAA GGAGCTGCCG TTATACTGTT CTGGGGGGTT GCGGTTTTTC      1080

TGGGACAACA AGTTTGACCA TGCAATGGTG GCTTTCCTGG ACTGTGTGCA GCAGTTCAAA      1140

GAAGAGGTTG AGAAAGGCGA GACACGTTTT TGTCTTCCCT ACAGGATGGA TGTGGAGAAA      1200

GGCAAGATTG AAGACACAGG AGGCAGTGGC GGCTCCTATT CCATCAAAAC CCAGTTTAAC      1260

TCTGAGGAGC AGTGGACAAA AGCTCTCAAG TTCATGCTGA CGAATCTTAA GTGGGGTCTT      1320
```

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GCTTGGGTGT CCTCACAATT TTATAACAAA TGACTTTTTT CCTTAGGGGG AGGTTTGCCT    1380

TAA                                                                 1383
```

(2) INFORMATION FOR SEQ ID NO:6:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 460 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: protein

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Glu Gly Ser Lys Thr Ser Asn Asn Ser Thr Met Gln Val Ser Phe
1               5                   10                  15

Val Cys Gln Arg Cys Ser Gln Pro Leu Lys Leu Asp Thr Ser Phe Lys
            20                  25                  30

Ile Leu Asp Arg Val Thr Ile Gln Glu Leu Thr Ala Pro Leu Leu Thr
        35                  40                  45

Thr Ala Gln Ala Lys Pro Gly Glu Thr Gln Glu Glu Glu Thr Asn Ser
    50                  55                  60

Gly Glu Glu Pro Phe Ile Glu Thr Pro Arg Gln Asp Gly Val Ser Arg
65                  70                  75                  80

Arg Phe Ile Pro Pro Ala Arg Met Met Ser Thr Glu Ser Ala Asn Ser
                85                  90                  95

Phe Thr Leu Ile Gly Glu Ala Ser Asp Gly Gly Thr Met Glu Asn Leu
            100                 105                 110

Ser Arg Arg Leu Lys Val Thr Gly Asp Leu Phe Asp Ile Met Ser Gly
        115                 120                 125

Gln Thr Asp Val Asp His Pro Leu Cys Glu Glu Cys Thr Asp Thr Leu
    130                 135                 140

Leu Gly Gln Leu Asp Thr Gln Leu Asn Val Thr Glu Asn Glu Cys Gln
145                 150                 155                 160

Asn Tyr Lys Arg Cys Leu Glu Ile Leu Glu Gln Met Asn Glu Asp Asp
                165                 170                 175

Ser Glu Gln Leu Gln Met Glu Leu Lys Glu Leu Ala Leu Glu Glu Glu
            180                 185                 190

Arg Leu Ile Gln Glu Leu Glu Asp Val Glu Lys Asn Arg Lys Ile Val
```

```
           195                    200                    205

  Ala Glu Asn Leu Glu Lys Val Gln Ala Glu Ala Glu Arg Leu Asp Gln
      210                    215                    220

  Glu Glu Ala Gln Tyr Gln Arg Glu Tyr Ser Glu Phe Lys Arg Gln Gln
  225                    230                    235                    240

  Leu Glu Leu Asp Asp Glu Leu Lys Ser Val Glu Asn Gln Met Arg Tyr
                245                    250                    255

  Ala Gln Thr Gln Leu Asp Lys Leu Lys Lys Thr Asn Val Phe Asn Ala
                260                    265                    270

  Thr Phe His Ile Trp His Ser Gly Gln Phe Gly Thr Ile Asn Asn Phe
          275                    280                    285

  Arg Leu Gly Arg Leu Pro Ser Val Pro Val Glu Trp Asn Glu Ile Asn
      290                    295                    300

  Ala Ala Trp Gly Gln Thr Val Leu Leu Leu His Ala Leu Ala Asn Lys
  305                    310                    315                    320

  Met Gly Leu Lys Phe Gln Arg Tyr Arg Leu Val Pro Tyr Gly Asn His
                325                    330                    335

  Ser Tyr Leu Glu Ser Leu Thr Asp Lys Ser Lys Glu Leu Pro Leu Tyr
                340                    345                    350

  Cys Ser Gly Gly Leu Arg Phe Phe Trp Asp Asn Lys Phe Asp His Ala
          355                    360                    365

  Met Val Ala Phe Leu Asp Cys Val Gln Gln Phe Lys Glu Glu Val Glu
  370                    375                    380

  Lys Gly Glu Thr Arg Phe Cys Leu Pro Tyr Arg Met Asp Val Glu Lys
  385                    390                    395                    400

  Gly Lys Ile Glu Asp Thr Gly Gly Ser Gly Gly Ser Tyr Ser Ile Lys
                405                    410                    415

  Thr Gln Phe Asn Ser Glu Glu Gln Trp Thr Lys Ala Leu Lys Phe Met
                420                    425                    430

  Leu Thr Asn Leu Lys Trp Gly Leu Ala Trp Val Ser Ser Gln Phe Tyr
      435                    440                    445

  Asn Lys  *   Leu Phe Ser Leu Gly Gly Gly Leu Pro
      450                    455                    460
```

(2) INFORMATION FOR SEQ ID NO:7:

```
    (1) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1240 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: not relevant

   (ii) MOLECULE TYPE: DNA (genomic)
```

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
ATGGAAGGGT CTAAGACGTC CAACAACAGC ACCATGCAGG TGAGCTTCGT GTGCCAGCGC      60

TGCAGCCAGC CCCTGAAACT GGACACGAGT TTCAAGATCC TGGACCGTGT CACCATCCAG     120

GAACTCACAG CTCCATTACT TACCACAGCC CAGGCGAAAC CAGGAGAGAC CCAGGAGGAA     180

GAGACTAACT CAGGAGAGGA GCCATTTATT GAAACTCCTC GCCAGGATGG TGTCTCTCGC     240

AGATTCATCC CCCCAGCCAG GATGATGTCC ACAGAAAGTG CCAACAGCTT CACTCTGATT     300

GGGGAGGCAT CTGATGGCGG CACCATCGAG AACCTCAGCC GAAGACTGAA GGTCACTGGG     360

GACCTTTTTG ACATCATGTC GGGCCAGACA GATGTGGATC ACCCACTCTG TGAGGAATGC     420

ACAGATACTC TTTTAGACCA GCTGGACACT CAGCTCAACG TCACTGAAAA TGAGTGTCAG     480

AACTACAAAC GCTGTTTGGA GATCTTAGAG CAAATGAATG AGGATGACAG TGAACAGTTA     540

CAGATGGAGC TAAAGGAGCT GGCACTAGAG GAGGAGAGGC TGATCCAGGA GCTGGAAGAC     600

GTGGAAAAGA ACCGCAAGAT AGTGGCAGAA AATCTCGAGA AGGTCCAGGC TGAGGCTGAG     660

AGACTGGATC AGGAGGAAGC TCAGTATCAG AGAGAATACA GTGAATTTAA ACGACAGCAG     720

CTGGAGCTGG ATGATGAGCT GAAGAGTGTT GAAAACCAGA TGCGTTATGC CCAGACGCAG     780

CTGGATAAGC TGAAGAAAAC CAACGTCTTT AATGCAACCT TCCACATCTG GCACACGTGGA     840

CAGTTTGGCA CAATCAATAA CTTCAGGCTG GGTCGCCTGC CCAGTGTTCC CGTGGAATGG     900

AATGAGATTA ATGCTGCTTG GGGCCAGACT GTGTTGCTGC TCCATGCTCT GGCCAATAAG     960

ATGGGTCTGA AATTTCAGAG ATACCGACTT GTTCCTTACG GAAACCATTC ATATCTGGAG    1020

TCTCTGACAG ACAAATCTAA GGATGGATGT GGAGAAAGGC AAGATTGAAG ACACAGGAGG    1080

CAGTGGCGGC TCCTATTCCA TCAAAACCCA GTTTAACTCT GAGGAGCAGT GGACAAAAGC    1140

TCTCAAGTTC ATGCTGACGA ATCTTAAGTG GGGTCTTGCT TGGGTGTCCT CACAATTTTA    1200

TAACAAATGA CTTTTTTCCT TAGGGGGAGG TTTGCCTTAA                          1240
```

:2) INFORMATION FOR SEQ ID NO:8

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 413 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: protein

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8

```
Met Glu Gly Ser Lys Thr Ser Asn Asn Ser Thr Met Gln Val Ser Phe
 1               5                  10                  15

Val Cys Gln Arg Cys Ser Gln Pro Leu Lys Leu Asp Thr Ser Phe Lys
            20                  25                  30

Ile Leu Asp Arg Val Thr Ile Gln Glu Leu Thr Ala Pro Leu Leu Thr
         35                  40                  45

Thr Ala Gln Ala Lys Pro Gly Glu Thr Gln Glu Glu Glu Thr Asn Ser
     50                  55                  60

Gly Glu Glu Pro Phe Ile Glu Thr Pro Arg Gln Asp Gly Val Ser Arg
 65                  70                  75                  80

Arg Phe Ile Pro Pro Ala Arg Met Met Ser Thr Glu Ser Ala Asn Ser
                85                  90                  95

Phe Thr Leu Ile Gly Glu Ala Ser Asp Gly Gly Thr Ile Glu Asn Leu
            100                 105                 110

Ser Arg Arg Leu Lys Val Thr Gly Asp Leu Phe Asp Ile Met Ser Gly
         115                 120                 125

Gln Thr Asp Val Asp His Pro Leu Cys Glu Glu Cys Thr Asp Thr Leu
 130                 135                 140

Leu Asp Gln Leu Asp Thr Gln Leu Asn Val Thr Glu Asn Glu Cys Gln
145                 150                 155                 160

Asn Tyr Lys Arg Cys Leu Glu Ile Leu Glu Gln Met Asn Glu Asp Asp
                165                 170                 175

Ser Glu Gln Leu Gln Met Glu Leu Lys Glu Leu Ala Leu Glu Glu Glu
            180                 185                 190

Arg Leu Ile Gln Glu Leu Glu Asp Val Glu Lys Asn Arg Lys Ile Val
         195                 200                 205

Ala Glu Asn Leu Glu Lys Val Gln Ala Glu Ala Glu Arg Leu Asp Gln
     210                 215                 220

Glu Glu Ala Gln Tyr Gln Arg Glu Tyr Ser Glu Phe Lys Arg Gln Gln
```

225   230   235   240

Leu Glu Leu Asp Asp Glu Leu Lys Ser Val Glu Asn Gln Met Arg Tyr
                   245   250   255

Ala Gln Thr Gln Leu Asp Lys Leu Lys Lys Thr Asn Val Phe Asn Ala
              260   265   270

Thr Phe His Ile Trp His Ser Gly Gln Phe Gly Thr Ile Asn Asn Phe
         275   280   285

Arg Leu Gly Arg Leu Pro Ser Val Pro Val Glu Trp Asn Glu Ile Asn
    290   295   300

Ala Ala Trp Gly Gln Thr Val Leu Leu Leu His Ala Leu Ala Asn Lys
305   310   315   320

Met Gly Leu Lys Phe Gln Arg Tyr Arg Leu Val Pro Tyr Gly Asn His
              325   330   335

Ser Tyr Leu Glu Ser Leu Thr Asp Lys Ser Lys Asp Gly Cys Gly Glu
         340   345   350

Arg Gln Asp * Arg His Arg Arg Gln Trp Arg Leu Leu Phe His Gln
         355   360   365

Asn Pro Val * Leu * Gly Ala Val Asp Lys Ser Ser Gln Val His
370   375   380

Ala Asp Glu Ser * Val Gly Ser Cys Leu Gly Val Leu Thr Ile Leu
385   390   395   400

 * Gln Met Thr Phe Phe Leu Arg Gly Arg Phe Ala Leu
              405   410

(2) INFORMATION FOR SEQ ID NO:9:

     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 1366 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: not relevant
         (D) TOPOLOGY: not relevant

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGGAAGGGT CTAAGACGTC CAACAACAGC ACCATGCAGG TGAGCTTCGT GTGCCAGCGC   60

TGCAGCCAGC CCCTGAAACT GGACACGAGT TTCAAGATCC TGGACCGTGT CACCATCCAG   120

GAACTCACAG CTCCATTACT TACCACAGCC CAGGCGAAAC CAGGAGAGAC CCAGGAGGAA   180

                                    11

GAGACTAACT CAGGAGAGGA GCCATTTATT GAAACTCCTC GCCAGGATGG TGTCTCTCGC   240

AGATTCATCC CCCCAGCCAG GATGATGTCC ACAGAAAGTG CCAACAGCTT CACTCTGATT   300

GGGGAGGCAT CTGATGGCGG CACCATGGAG AACCTCAGCC GAAGACTGAA GGTCACTGGG   360

GACCTTTTTG ACATCATGTC GGGCCAGACA GATGTGGATC ACCCACTCTG TGAGGAATGC   420

ACAGATACTC TTTTAGACCA GCTGGACACT CAGCTCAACG TCACTGAAAA TGAGTGTCAG   480

AACTACAAAC GCTGTTTGGA GATCTTAGAG CAAATGAATG AGGATGACAG TGAACAGTTA   540

CAGATGGAGC TAAAGGAGCT GGCACTAGAG GAGGAGAGGC TGATCCAGGA GCTGGAAGAC   600

GTGGAAAAGA ACCGCAAGAT AGTGGCAGAA AATCTCGAGA AGGTCCAGGC TGAGGCTGAG   660

AGACTGGATC AGGAGGAAGC TCAGTATCAG AGAGAATACA GTGAATTTAA ACGACAGCAG   720

CTGGAGCTGG ATGATGAGCT GAAGAGTGTT GAAAACCAGA TGCGTTATGC CCAGACGCAG   780

CTGGATAAGC TGAAGAAAAC CAACGTCTTT AATGCAACCT TCCACATCTG GCACAGTGGA   840

CAGTTTGGCA CAATCAATAA CTTCAGGCTG GGTCGCCTGC CCAGTGTTCC CGTGGAATGG   900

AATGAGATTA ATGCTGCTTG GGGCCAGACT GTGTTGCTGC TCCATGCTCT GGCCAATAAG   960

ATGGGTCTGA AATTTCAGAG ATACCGACTT GTTCCTTACG GAAACCATTC ATATCTGGAG   1020

TCTCTGACAG ACAAATCTAA GGCTGAAGTG CAATGGCATG ATCTCGGCTT ACTGCAACCT   1080

CCGCCTCCCG GGTTCAAGCA ATTCCCCTGC CTCAGCCTCC TGAGTATCTG GGATTACAGG   1140

CATGCACCAC CACGCCCGGC TAATTAGGAT GGATGTGGAG AAAGGCAAGA TTGAAGACAC   1200

AGGAGGCAGT GGCGGCTCCT ATTCCATCAA AACCCAGTTT AACTCTGAGG AGCAGTGGAC   1260

AAAAGCTCTC AAGTTCATGC TGACGAATCT TAAGTGGGGT CTTGCTTGGG TGTCCTCACA   1320

ATTTTATAAC AAATGACTTT TTTCCTTAGG GGGAGGTTTG CCTTAA   1366

(2) INFORMATION FOR SEQ ID NO:10

        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 455 amino acids
              (B) TYPE: amino acid
              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10

Met Glu Gly Ser Lys Thr Ser Asn Asn Ser Thr Met Gln Val Ser Phe
 1   5   10   15

                                    12

Val Cys Gln Arg Cys Ser Gln Pro Leu Lys Leu Asp Thr Ser Phe Lys
                20   25   30

Ile Leu Asp Arg Val Thr Ile Gln Glu Leu Thr Ala Pro Leu Leu Thr
            35   40   45

Thr Ala Gln Ala Lys Pro Gly Glu Thr Gln Glu Glu Glu Thr Asn Ser
        50   55   60

Gly Glu Glu Pro Phe Ile Glu Thr Pro Arg Gln Asp Gly Val Ser Arg
    65   70   75   80

Arg Phe Ile Pro Pro Ala Arg Met Met Ser Thr Glu Ser Ala Asn Ser
                85   90   95

Phe Thr Leu Ile Gly Glu Ala Ser Asp Gly Gly Thr Met Glu Asn Leu
            100   105   110

Ser Arg Arg Leu Lys Val Thr Gly Asp Leu Phe Asp Ile Met Ser Gly
        115   120   125

Gln Thr Asp Val Asp His Pro Leu Cys Glu Glu Cys Thr Asp Thr Leu
    130   135   140

Leu Asp Gln Leu Asp Thr Gln Leu Asn Val Thr Glu Asn Glu Cys Gln
145   150   155   160

Asn Tyr Lys Arg Cys Leu Glu Ile Leu Glu Gln Met Asn Glu Asp Asp
                165   170   175

Ser Glu Gln Leu Gln Met Glu Leu Lys Glu Leu Ala Leu Glu Glu Glu
            180   185   190

Arg Leu Ile Gln Glu Leu Glu Asp Val Glu Lys Asn Arg Lys Ile Val
        195   200   205

Ala Glu Asn Leu Glu Lys Val Gln Ala Glu Ala Glu Arg Leu Asp Gln
    210   215   220

Glu Glu Ala Gln Tyr Gln Arg Glu Tyr Ser Glu Phe Lys Arg Gln Gln
225   230   235   240

Leu Glu Leu Asp Asp Glu Leu Lys Ser Val Glu Asn Gln Met Arg Tyr
                245   250   255

Ala Gln Thr Gln Leu Asp Lys Leu Lys Lys Thr Asn Val Phe Asn Ala
            260   265   270

Thr Phe His Ile Trp His Ser Gly Gln Phe Gly Thr Ile Asn Asn Phe
        275   280   285

Arg Leu Gly Arg Leu Pro Ser Val Pro Val Glu Trp Asn Glu Ile Asn
    290   295   300

Ala Ala Trp Gly Gln Thr Val Leu Leu Leu His Ala Leu Ala Asn Lys

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305                    310                    315                    320

Met Gly Leu Lys Phe Gln Arg Tyr Arg Leu Val Pro Tyr Gly Asn His
                   325                    330                335

Ser Tyr Leu Glu Ser Leu Thr Asp Lys Ser Lys Ala Glu Val Gln Trp·
                340                    345                    350

His Asp Leu Gly Leu Leu Gln Pro Pro Pro Pro Gly Phe Lys Gln Phe
            355                    360                    365

Pro Cys Leu Ser Leu Leu Ser Ile Trp Asp Tyr Arg His Ala Pro Pro
        370                    375                    380

Arg Pro Ala Asn  *  Asp Gly Cys Gly Glu Arg Gln Asp  *  Arg His
385                    390                    395                    400

Arg Arg Gln Trp Arg Leu Leu Phe His Gln Asn Pro Val  *  Leu  *
                405                    410                    415

Gly Ala Val Asp Lys Ser Ser Gln Val His Ala Asp Glu Ser  *  Val
                420                    425                    430

Gly Ser Cys Leu Gly Val Leu Thr Ile Leu  *  Gln Met Thr Phe Phe
            435                    440                    445

Leu Arg Gly Arg Phe Ala Leu
    450                    455
```

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1509 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: not relevant
        (D) TOPOLOGY: not relevant

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
ATGGAAGGGT CTAAGACGTC CAACAACAGC ACCATGCAGG TGAGCTTCGT GTGCCAGCGC      60

TGCAGCCAGC CCCTGAAACT GGACACGAGT TTCAAGATCC TGGACCGTGT CACCATCCAG     120

GAACTCACAG CTCCATTACT TACCACAGCC CAGGCGAAAC CAGGAGAGAC CCAGGAGGAA     180

GAGACTAACT CAGGAGAGGA GCCATTTATT GAAACTCCTC GCCAGGATGG TGTCTCTCGC     240

AGATTCATCC CCCCAGCCAG GATGATGTCC ACAGAAAGTG CCAACAGCTT CACTCTGATT     300

GGGGAGGCAT CTGATGGCGG CACCATGGAG AACCTCAGCC GAAGACTGAA GGTCACTGGG     360
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```
GACCTTTTTG ACATCATGTC GGGCCAGACA GATGTGGATC ACCCACTCTG TGAGGAATGC    420

ACAGATACTC TTTTAGACCA GCTGGACACT CAGCTCAACG TCACTGAAAA TGAGTGTCAG    480

AACTACAAAC GCTGTTTGGA GATCTTAGAG CAAATGAATG AGGATGACAG TGAACAGTTA    540

CAGATGGAGC TAAAGGAGCT GGCACTAGAG GAGGAGAGGC TGATCCAGGA GCTGGAAGAC    600

GTGGAAAAGA ACCGCAAGAT AGTGGCAGAA AATCTCGAGA AGGTCCAGGC TGAGGCTGAG    660

AGACTGGATC AGCAGGAAGC TCAGTATCAG AGAGAATACA GTGAATTTAA ACGACAGCAG    720

CTGGAGCTGG ATGATGAGCT GAAGAGTGTT GAAAACCAGA TGCGTTATGC CCAGACGCAG    780

CTGGATAAGC TGAAGAAAAC CAACGTCTTT AATGCAACCT TCCACATCTG GCACAGTGGA    840

CAGTTTGGCA CAATCAATAA CTTCAGGCTG GGTCGCCTGC CCAGTGTTCC CGTGGAATGG    900

AATGAGATTA ATGCTGCTTG GGGCCAGACT GTGTTGCTGC TCCATGCTCT GGCCAATAAG    960

ATGGGTCTGA AATTTCAGAG ATACCGACTT GTTCCTTACG GAAACCATTC ATATCTGGAG   1020

TCTCTGACAG ACAAATCTAA GGAGCTGCCG TTATACTGTT CTGGGGGGTT GCGGTTTTTC   1080

TGGGACAACA AGGCTGAAGT GCAATGGCAT GATCTCGGCT TACTGCAACC TCCGCCTCCC   1140

GGGTTCAAGC AATTCCCCTG CCTCAGCCTC CTGAGTATCT GGGATTACAG GCATGCACCA   1200

CCACGCCCGG CTAATTAGTT TGACCATGCA ATGGTGGCTT TCCTGGACTG TGTGCAGCAG   1260

TTCAAAGAAG AGGTTGAGAA AGGCGAGACA CGTTTTTGTC TTCCCTACAG GATGGATGTG   1320

GAGAAAGGCA AGATTGAAGA CACAGGAGGC AGTGGCGGCT CCTATTCCAT CAAAACCCAG   1380

TTTAACTCTG AGGAGCAGTG GACAAAAGCT CTCAAGTTCA TGCTGACGAA TCTTAAGTGG   1440

GGTCTTGCTT GGGTGTCCTC ACAATTTTAT AACAAATGAC TTTTTTCCTT AGGGGGAGGT   1500

TTGCCTTAA                                                            1509
```

(2) INFORMATION FOR SEQ ID NO:12:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 503 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: protein

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Glu Gly Ser Lys Thr Ser Asn Asn Ser Thr Met Gln Val Ser Phe
 1   5   10   15

```
Val Cys Gln Arg Cys Ser Gln Pro Leu Lys Leu Asp Thr Ser Phe Lys
            20                      25                  30

Ile Leu Asp Arg Val Thr Ile Gln Glu Leu Thr Ala Pro Leu Leu Thr
        35                      40                  45

Thr Ala Gln Ala Lys Pro Gly Glu Thr Gln Glu Glu Thr Asn Ser
    50                      55                  60

Gly Glu Glu Pro Phe Ile Glu Thr Pro Arg Gln Asp Gly Val Ser Arg
  65                      70                  75                  80

Arg Phe Ile Pro Pro Ala Arg Met Met Ser Thr Glu Ser Ala Asn Ser
                85                      90                  95

Phe Thr Leu Ile Gly Glu Ala Ser Asp Gly Gly Thr Met Glu Asn Leu
            100                     105                 110

Ser Arg Arg Leu Lys Val Thr Gly Asp Leu Phe Asp Ile Met Ser Gly
        115                     120                 125

Gln Thr Asp Val Asp His Pro Leu Cys Glu Glu Cys Thr Asp Thr Leu
    130                     135                 140

Leu Asp Gln Leu Asp Thr Gln Leu Asn Val Thr Glu Asn Glu Cys Gln
145                     150                     155                 160

Asn Tyr Lys Arg Cys Leu Glu Ile Leu Glu Gln Met Asn Glu Asp Asp
                165                     170                 175

Ser Glu Gln Leu Gln Met Glu Leu Lys Glu Leu Ala Leu Glu Glu Glu
            180                     185                 190

Arg Leu Ile Gln Glu Leu Glu Asp Val Glu Lys Asn Arg Lys Ile Val
        195                     200                 205

Ala Glu Asn Leu Glu Lys Val Gln Ala Glu Ala Glu Arg Leu Asp Gln
210                     215                 220

Gln Glu Ala Gln Tyr Gln Arg Glu Tyr Ser Glu Phe Lys Arg Gln Gln
225                     230                 235                 240

Leu Glu Leu Asp Asp Glu Leu Lys Ser Val Glu Asn Gln Met Arg Tyr
                245                     250                 255

Ala Gln Thr Gln Leu Asp Lys Leu Lys Lys Thr Asn Val Phe Asn Ala
            260                     265                 270

Thr Phe His Ile Trp His Ser Gly Gln Phe Gly Thr Ile Asn Asn Phe
        275                     280                 285

Arg Leu Gly Arg Leu Pro Ser Val Pro Val Glu Trp Asn Glu Ile Asn
    290                     295                 300

Ala Ala Trp Gly Gln Thr Val Leu Leu Leu His Ala Leu Ala Asn Lys
```

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```
305                    310                    315                    320

Met Gly Leu Lys Phe Gln Arg Tyr Arg Leu Val Pro Tyr Gly Asn His
                325                    330                    335

Ser Tyr Leu Glu Ser Leu Thr Asp Lys Ser Lys Glu Leu Pro Leu Tyr
                340                    345                    350

Cys Ser Gly Gly Leu Arg Phe Phe Trp Asp Asn Lys Ala Glu Val Gln
            355                    360                    365

Trp His Asp Leu Gly Leu Leu Gln Pro Pro Pro Gly Phe Lys Gln
        370                    375                    380

Phe Pro Cys Leu Ser Leu Leu Ser Ile Trp Asp Tyr Arg His Ala Pro
385                    390                    395                    400

Pro Arg Pro Ala Asn  *  Phe Asp His Ala Met Val Ala Phe Leu Asp
                405                    410                    415

Cys Val Gln Gln Phe Lys Glu Glu Val Glu Lys Gly Glu Thr Arg Phe
                420                    425                    430

Cys Leu Pro Tyr Arg Met Asp Val Glu Lys Gly Lys Ile Glu Asp Thr
        435                    440                    445

Gly Gly Ser Gly Gly Ser Tyr Ser Ile Lys Thr Gln Phe Asn Ser Glu
450                    455                    460

Glu Gln Trp Thr Lys Ala Leu Lys Phe Met Leu Thr Asn Leu Lys Trp
465                    470                    475                    480

Gly Leu Ala Trp Val Ser Ser Gln Phe Tyr Asn Lys  *  Leu Phe Ser
                485                    490                    495

Leu Gly Gly Gly Leu Pro  *
            500
```

(2) INFORMATION FOR SEQ ID NO:13:

  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 20 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: not relevant

  (ii) MOLECULE TYPE: DNA (genomic)

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCGACTTGTT CCTTACGGAA   20

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: not relevant

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGTGTCTCGC CTTTCTCAAC   20

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: not relevant

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TGCGGTTTTT CTGGGACAAC   20

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: not relevant

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CAAGCAAGAC CCCACTTAAG   20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 14 amino acids
    (B) TYPE: amino acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Glu Gly Ser Lys Thr Ser Asn Asn Ser Thr Met Gln Val
1   5   10

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 16 amino acids
    (B) TYPE: amino acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Cys Val Gln Gln Phe Lys Glu Glu Val Glu Lys Gly Glu Thr Arg Phe
1   5   10   15

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 20 amino acids
    (B) TYPE: amino acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Glu Leu Phe Arg Asp Gly Val Asn Trp Gly Arg Ile Val Ala Phe Phe
1   5   10   15

Glu Phe Gly Gly
       20

(2) INFORMATION FOR SEQ ID NO:20:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 20 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (iii) HYPOTHETICAL: NO

   (iv) ANTI-SENSE: NO

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

    Glu Leu Phe Arg Asp Gly Val Asn Trp Gly Arg Ile Val Ala Phe
Phe
    1   5   10   15

    Ser Phe Gly Gly
          20

(2) INFORMATION FOR SEQ ID NO:21:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 20 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (iii) HYPOTHETICAL: NO

   (iv) ANTI-SENSE: NO

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

    Glu Leu Phe Arg Asp Gly Val Asn Trp Ala Arg Ile Val Ala Phe Phe
    1   5   10   15

    Glu Phe Gly Gly
          20

(2) INFORMATION FOR SEQ ID NO:22:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 20 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Glu Leu Phe Arg Asp Gly Ala Ile Leu Gly Arg Ile Val Ala Phe Phe
1   5   10   15

Ser Phe Gly Gly
            20

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/68; A61K 48/00; C07H 21/04
US CL : 435/6; 514/44; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6; 514/44; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C.	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,858,669 A (LEVINE) 12 January 1999 (12.01.1999), see entire document.	1-4
Y, P	AITA et al. Cloning and Genomic Organization of Beclin 1, a candidate tumor suppressor gene on chromosome 17q21. Genomics. 1999, Vol 59, pages 59-65, entire document..	1-4
X, P	LIANG et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature. December 1999, Vol 402, pages 672-676, entire document.	1-4
Y	FRIDMAN et a.l 22 Genes from Chromosome 17q21: Cloning, Sequencing and Characterization of mutations in breast cancer families and tumors. Genomics. 1995, Vol 25, pages 256-263, entire document.	1-4
Y	ROMMENS et al. Generation of a transcription map at the HSD17B locus Centromeric to BRCA1 at 17q21. Genomics. 1995, Vol 28, pages 530-542, entire document.	1-4

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"B"	earlier application or patent published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 June 2000 (22.06.2000)	11 JUL 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Jeanine Enewold Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

| **Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)** |

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

| **Box II   Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)** |

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-4

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.
                    ☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I- Claims 1-4, drawn to a method for treating cancer by administering beclin to restore cell growth control.

Group II- Claims 5-15 drawn to a method for inhibiting viral replication comprising inducing expression of beclin with virus infected cells.

Group III- Claims 16-37, 80-97, 99, 101-102 drawn to a method of diagnosing a predisposition to carcinoma comprising determining whether a subjects allele or regulatory regions are deleted or different compared to the wild-type.

Group IV- Claims 38-42 drawn to a method for diagnosing a predisposition to cancer in a subject by determining whether the subject has abnormal subcellular distribution of beclin.

Group V- Claims 43-48 drawn to a method of diagnosing a predisposition to carcinoma in a subject by contact the cells with antibodies specific to beclin.

Group VI- Claims 49-58, 63-75, 103-104 drawn to a to a nucleic acid encoding wild-type human beclin, a nucleic acid encoding a mutant human beclin, a method of producing a polypeptide by expressing the nucleic acid, and a pharmaceutical composition comprising the nucleic acid encoding beclin.

Group VII- Claims 59-62, drawn to human beclin proteins.

Group VIII- Claims 104 drawn to a pharmaceutical composition comprising the polypeptide beclin.

Group IX- Claims 76-79 drawn to oligonucleotides which distinguish nucleic acids from mutant and wild-type beclin.

Group X- Claim 98 drawn to a method of inhibiting cell proliferation with a nucleic acid.

Group XI- Claims 100, 105 drawn to a method treating cancer with the nucleic acid.

Group XII- Claims 105 drawn to a method of treating cancer with the pharmaceutical composition containing a polypeptide.

The inventions listed as Groups I-XIV do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons.

The Beclin protein appears to be the technical feature which links the inventions of Groups I-XIV. However, unity of invention is lacking because Friedman et al (Genomic, Vol 25, pg. 256-263, 1995) teaches the nucleic acid sequence of Beclin. Moreover, Rommens et al (Genomic, Vol. 28, pg. 530-542, 1995) teaches a partial ORF with a translated protein sequence which appears to be Beclin. Furthermore, Liang (J. of Virology, Vol. 72, no. 11, pg. 8586-8596, November 1998), and Gordon (Biophysical Journal, Vol. 74, page. 2702-2713, May 1998) both teach that Beclin was characterized. Thus, the inventions are not linked by a special technical feature as defined by PCT Rule 13.2, and therefore lack unity.

Continuation of B. FIELDS SEARCHED Item 3: Medline, Biosis, Caplus, Embase, East
   beclin, cancer, proliferative